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We have continued our eforts to characterize the immunological propeties of the V1/V2 domain of HIV-1 gp120. Studies with human sera and with antibodies isolated from immunized animals have shown that the V1/V2 domain contains highly conserved epitopes that can act as potent neutralizing targets for a broad range of primary HIV-1 isolates. We have prepared two prototype immunogens, based on the Case-A2 and SF-162 isolates. The Case-A2 isolate contains the clade B V2 consensus sequence, and this antigen induces highly crossreactive anti-V1/V2 antibodies in both rats and macaques that possess potent cross-neutralizing activity for several primary HIV-1 isolates. Our studies to date have shown that while the V1/V2 domain remains an intriguing target for viral neutralization and a candidate for HIV vaccine development, additional modification of the V1/V2 fusion proteins is needed to improve their specificities and efficiencies as vaccines capable of inducing relevant neutralizing responses.

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FOREWORD

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Introduction

Progress towards an effective HIV vaccine has been stymied by the inability of current vaccines to induce sufficiently high titers of antibodies capable of neutralizing primary HIV-1 isolates. Although some sera of HIV-1 infected patients contain such antibodies, these sera are rare, and the nature of the epitopes that mediate this activity is unknown. The major neutralization domains identified for laboratory-adapted viruses, the V3 loop (8) and CD4-binding site of gp120 (2, 6, 16, 17, 20), do not appear to be potent neutralization targets for primary viruses. One study showed that depletion of anti-V3 antibodies from three human sera resulted in the removal of the majority of neutralizing activity for MN virus, but had no significant effect on neutralizing activities for two primary isolates (21); a second study suggested that the V3 loop in M-tropic viruses is cryptic and unavailable for antibody binding (1). Most MAbs against the CD4-binding site with potent neutralizing activities against lab strains have little if any neutralizing activity for primary, macrophage-tropic isolates (3, 12, 19); in some cases such MAbs have been reported to enhance infection by some primary isolates (18, 19). Whereas standard rgp120-based immunogens have not been able to induce protective responses, a recent report indicates that mice immunized with fusion-activated forms of HIV-1 Env proteins can mount a potent, cross-protective humoral response to primary isolates (10). This indicates that highly conserved protective epitopes do exist on the surface of virions, but that they may not be expressed in the standard immunogens currently being used as HIV vaccines.

Considerable evidence suggests that the V1/V2 domain of gp120 is one of the major antigenic regions exposed on the surface of the virion. Whereas most anti-V2 MAbs that have been described possess weak neutralizing activities for both laboratory strains and primary viruses (4, 5, 11, 13, 14), we have isolated a chimpanzee MAb, C108G, directed against a type-specific glycan-dependent epitope in the V2 region, that possesses remarkably potent neutralization activity for both T-tropic and M-tropic viruses that bear the epitope (7, 23, 24).

In order to allow the characterization of the immunological structure of the V1/V2 domain, we have utilized a novel fusion protein system to express the isolated V1/V2 domain of HIV gp120. These epitopes have centered on the Case-A2 V1/V2 glycoprotein, a recombinant fusion protein which contains the clade B consensus sequence in the central region of the V2 domain. We have shown that this protein is highly immunogenic in rats, and induces crossreactive anti-V1/V2 antibodies which possess potent neutralizing activities for both T cell-tropic and macrophage-tropic viruses, including several non-clade B primary isolates. More recently we showed that similar responses could also be obtained in rhesus macaques immunized with the Case-A2 V1/V2 fusion protein, in the presence of a three component RAS adjuvant, composed of MPL, TDM and CWS (Ribi Immunochemical). Sera obtained at 1 week after the boost with the V1/V2 fusion protein contained relatively high titers against the respective immunogens, and these sera also recognized a number of recombinant gp120s and a variety of heterologous V1/V2 fusion proteins.

Progress Report: Development of an HIV vaccine based on the V1/V2 domain of gp120

Whereas it is known that some human immune sera possess potent neutralizing activities for primary viruses, the identity of the target epitopes mediating this neutralization is unknown, and currently available immunogens have not been able to induce such activities. Recent evidence from our laboratory suggests that the V1/V2 domain of HIV-1 gp120 contains epitopes that are potent neutralization targets for macrophage-tropic HIV-1 isolates (7, 15). The objectives of this study were to elicit V1/V2-specific antibodies by immunization with a recombinant protein that expressed the isolated V1/V2 domain and to determine the breadth and potency of HIV-1 neutralization by these antibodies.

Expression and characterization of isolated V1/V2 domain

The V1/V2 domain of a clinical HIV-1 isolate (Case-A2) was expressed as a fusion glycoprotein in CHO cells. This sequence was selected since the central portion of its V2 domain was almost identical to the clade B consensus sequence of this region (22). The carrier sequence chosen was derived from the N-terminal domain of the murine leukemia virus SU protein, gp70 (9). The Case-A2 V1/V2 protein was purified by affinity chromatography on a Ni-NTA column, utilizing a His6 tag incorporated near the N-terminus of the carrier gp70 sequence. The protein was shown by SDS-PAGE to be >90% pure.

Despite the apparent purity of the V1/V2 fusion protein, a radioimmunoprecipitation analysis of the labeled protein demonstrated the presence of two conformational forms of the antigen, which differed both in their reactivity with different monoclonal antibodies and their mobility on SDS gels when analyzed under nonreducing conditions. Both bands were recognized by K10A11, an antibody directed against a site in the gp70-derived carrier domain, and by C9B6, a mab directed against a linear V2 epitope. The upper band was recognized by K19B3, a mab directed against a conserved V1/V2 epitope, while the lower band was recognized by SC258 and 697D, mouse and human mabs directed against conformation V2 epitopes, as well as by several other mouse and human mabs directed against native conformational epitopes and by a number of human sera. This preferential recognition of lower band by the human antibodies suggested that it represented the correctly folded form, while the upper band represented an alternative, presumably nonnative conformation. The two bands coalesced after reduction of disulfide bonds with DTT, confirming that they represented distinct disulfide-bonded conformers. We were able to fractionate these forms by affinity chromatography on a column to which monoclonal antibody SC258 was immobilized. The K19-reactive form was present in the flow through, while the native form was eluted by low pH buffer.

Immunization of monkeys with purified V1/V2 fusion proteins

Three pigtailed macaques (# 6876, 6876 and 7026) were immunized with purified Case-A2 V1/V2 protein in the presence of Ribi RAS triple adjuvant (Monophosphoryl Lipid A {MPL}, Trehalose Dicorynomycolate {TDM}, and Cell Wall Skeleton {CWS} at initial doses of 25 μg/kg. Animal 6876 was boosted with the unfractionated antigen at 5 μg/kg, while the other two V1/V2-

immunized animals (#7014 and #7026) were boosted with the SC238 Mab affinity-purified fraction of the V1/V2 protein. A fourth animal (#6874) was immunized with an equivalent amount of the gp70-related carrier sequence. The animals were bled prior to each immunization and at weekly intervals following each immunization. All three animals generated significant antibody titers against the fusion protein immediately after the first boost. These titers decayed after several months, and a potent anamnestic response was observed in all animals following the second boost.

Both monkeys immunized with the purified Case-A2-V1/V2 fusion protein produced antibodies that reacted with heterologous gp120s as well as with V1/V2 domains derived from *env* sequences of a number of unrelated HIV-1 isolates, including one Thai clade E sequence. These antibodies appeared to be recognizing common conserved sequences, as evidenced by the fact that almost all of the reactivity of antibodies induced by the Case-A2 V1/V2 sequence for two unrelated recombinant Env proteins (derived from the Ba-L and 451 isolates) was absorbed by the heterologous SF162 V1/V2 protein. In initial assays, we found that sera of the three animals immunized with the V1/V2 protein, but not that of the control animal, were able to neutralize the macrophage-tropic NL-HX-ADA virus. These results indicate that the Case-A2 V1/V2 fusion protein was able to induce crossreactive antibodies against native gp120 epitopes.

The V1/V2-specific IgG fraction of the immune sera was fractionated by sequential immunoaffinity chromatography on a column containing the immobilized gp70-related carrier protein, followed by passage over a column containing the complete V1/V2 fusion protein. This resulted in removal of >95% of all V1/V2-reactive antibodies. A portion of the V1/V2-specific antibodies bound to the second column were recovered by sequential elution with low pH buffers followed by elution with buffer containing 8M guanidine hydrochloride. After extensive buffer exchange, the isolated antibodies were quantitated and tested for HIV-1 neutralizing activities.

All of the eluted antibody fractions possessed neutralizing activities for a number of macrophage-tropic isolates, with the lower pH and GuHCl antibody fraction generally being more potent than that eluted at pH 2.4. In addition to NL-HX-ADA, viruses neutralized included Ba-L, a recombinant derived from NL-HX-ADA that contained the Case-A2 V1/V2 domain, and a clade C primary isolate from Malawi, 92MW965C.

A surprising result was that in contrast to the efficient neutralization of NL-HX-ADA and NL-Case-A2-ADA, these antibodies did not neutralize two related molecular recombinants that contained the identical V1/V2, but with T cell-tropic V3-V5 regions. This result suggested that the neutralizing activity of these antibodies was specific for M-tropic isolates. This supports a model in which the key V1/V2 epitopes that are targetted by these antibodies function specifically in CCR5-dependent infections, either by virtue of a direct interaction with the CCR5 receptor, or as a result of a conformational structure that is specific for macrophage-tropic envelope proteins.

These studies have demonstrated that our Case-A2 V1/V2 fusion protein is capable of inducing potenct neutralizing antibodies against native epitopes in the V1/V2 domain. However, the yield of this class of antibodies in the resulting immune sera has been low, and the majority of antibodies

produced appeared to be directed against epitopes that do not mediate neutralization and that may be carried on non-native forms of the immunogen. Our challenge is to learn how to modify the immunogen and/or immunization protocols so that the focus of the immune response is directed against the neutralizing targets. We are currently performing mutagenesis studies of our V1/V2 fusion protein to define the role of individual residues in determining folding, immunoreactivity and immunogenicity of this protein. Ideally, we would like to map the different classes of epitopes. This would allow us to hopefully modify the immunogen so that the deletorious epitopes are eliminated, while retaining and perhaps enhance the immunogenicity of the protective epitopes. The broad crossreactivity and potent cross-neutralizing activities we have been able to induce in rodents and macaques with our current form of the V1/V2 immunogen is very encouraging, and suggests that this vaccine, and improved versions derived from the current immunogen, may induce protective responses in humans as well.

Key Research Accomplishments

-Development of expression system system for presenting native domains of gp120
-Characterization of human humoral response to the V1/V2 domain of gp120
-Demonstration that human antibodies directed against conserved epitopes in the V1/V2 domain possess broad neutralizing activities
-Identification of multiple conformational states of the V1/V2 domain
-Isolation of monoclonal antibodies specific for different V1/V2 conformers
-Development of a V1/V2 miniprotein suitable as an immunogen
-Characterization of immune response of rodents and primates to a V1/V2 antigen
-Demonstration that antibodies derived by immunization with a V1/V2 immunogen recognize highly conserved epitopes and are capable of neutralizing primary HIV-1 isolates

Reportable outcomes

Publications

Honnen, W.J., Z.Wu, S.C. Kayman and A. Pinter. 1996. Potent neutralization of a macrophage-tropic HIV-1 isolate by antibodies against the V1/V2 domain of gp120. Vaccines 1996: Molecular Approaches to the Contrc of Infectious Diseases. pp. 289-297.

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Kayman, S., A. Pinter, O. Trochev, W. Honnen, and F. Zhao. 1999. Retrovirus particle display for immunogen optimization. NIH/AVRC Workshop, Bethesda MD, May 3-5, 1999.

Patent applications

A. Pinter. HIV-1 gp120 V1/V2 domain epitopes capable of generating neutralizing antibodies. Patent filed Sept. 8, 1997.

Development of cell lines expressing HIV gp120 protein domains

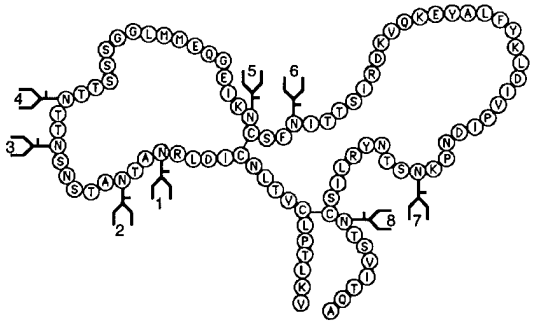
A large number of expression plasmids and cell lines producing native V1/V2 domain fusion proteins, including a panel of mutant V1/V2 sequences, have been generated. A partial list includes stable CHO cell lines expressing the following proteins:

Construct #	
	clade A V1/V2 miniproteins
849	UG31.4 A
855	Br.92.20.04 A
627	Ug 37.8 A
629	RW20.5 A
858	Ug.92.37.08 A
	clade B V1/V2 miniproteins
493	HXB2 B
562	B23 B
564	CASE.A2 B
600	Br 20.4 B
601	Th 26.6, B
602	Th 14.12 B
742	89.6 B
762	MN-ST B
764	SF162 B

Construct #	
	clade D V1/V2 miniproteins
628	Ug 21.9 D
	clade E V1/V2 miniproteins
857	Th.92.22.04 E
	clade B V1/V2 deletions
874	Stem-Only
875	Stemless no Glycan Case A2
876	Stemless with Glycan Case A2
877	Stemless no Glycan SF162
878	Stemless with Glycan SF162
	clade B V1/V2 glycan mutants
	All eight CaseA2 sites
	All four SF162 sites
	clade B V1/V2/TEV cleavage sites
623	V1/V2 CaseA2 + TEV
850	V1/V2 SF162 + TEV

Partial list of V1/V2 sequences and mutants available as fusion proteins

CaseA2 V1/V2 structure (p565)



wild type

gs1⁻ TÆA	gs2⁻ TÆA
gs1⁻ 2⁻ TÆA	gs3⁻ TÆA
gs3⁻ 4⁻ TÆA	gs4⁻ TÆA
gs1⁻ 2⁻ 3⁻ 4⁻ TÆA	gs5⁻ SÆA
gs6⁻ TÆA	gs6⁻ NÆA
gs6⁻ NÆQ	gs7⁻ TÆA
gs8⁻ NÆQ	gs8⁻ SÆA

Structure of the CaseA2 V1/V2 indicating each of the N-linked glycosylation sites, and list of specific single and multiple glycosylation site mutants already constructed for this protein.

Conclusions and Future Directions

Our studies during the past few years have confirmed the potency of antibodies against native epitopes in the V1/V2 domain, and have demonstrated that our Case-A2 V1/V2 fusion protein is capable of inducing such antibodies. However, the yield of this class of antibodies has been low, while the majority of the antibodies produced were directed against epitopes that do not mediate neutralization and that may be carried on non-native forms of the immunogen. In addition, we have found that a subfraction of the antibodies induced possesses enhancing activity against at least one primary isolate. Our challenge during the coming years is to define the relevant neutralizing and enhancing epitopes, and to learn how to modify the immunogen and/or immunization protocols so that the focus of the immune response is directed specifically towards the neutralizing targets.

A key goal of this approach is to develop means of improving these V1/V2 immunogens to focus the humoral responses towards the relevant epitopes that induce the most potent neutralizing responses. This will involve evaluation of alternate strategies to either remove or modify the gp70-derived fusion sequences, to avoid the generation of antibodies against the carrier sequences. More importantly, our studies have shown that the V1/V2 domain is an immunologically complex structure. The most potently neutralizing antibodies are directed against the conserved conformational epitopes, and these are the most relevant ones for protection. A second class of antibodies are directed against the T15K V2 linear epitope. Whereas these also neutralize many viruses, they are less potent. One possibility is that mutating this sequence may increase the immunogenicity of the conformational epitopes. Previous studies with monoclonal antibodies to V1/V2 have shown that most of these have little, if any neutralizing activity. Such antibodies, if present in excess may be deletorious, since they may block the interaction of the neutralizing antibodies, thereby inhibiting their activities. Finally, we have found in some human sera the presence of anti-V1/V2 antibodies that actually enhance the infection by some strains of HIV-1. Such antibodies could be harmful if induced in response to immunization.

We have initiated mutagenesis studies of our V1/V2 fusion protein to define the role of individual residues in determining folding, immunoreactivity and immunogenicity of this protein. Iniitial targets being addressed are individual N-linked glycosylation sites. We are also expressing smaller subdomains of the V1/V2 region, including proteins containing only the conserved stem of the V1/V2 domain and proteins in which the conserved stem sequences have been deleted. These studies should help us define the relevant neutralizing epitopes, and hopefully would allow us to eliminate deletorious epitopes, while retaining and perhaps enhance the immunogenicity of the protective epitopes. The broad crossreactivity and potent cross-neutralizing activities we have been able to induce in rodents and macaques with our current vaccine is very encouraging, and suggests that this vaccine, and improved versions derived from the current immunogen, may induce protective responses in humans as well.

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Potent Neutralization of a Macrophage-tropic HIV-1 Isolate by Antibodies against the V1/V2 Domain of HIV-1 gp120

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A number of studies with monoclonal antibodies (MAbs) have shown that the V1/V2 domain of human immunodeficiency virus type-1 (HIV-1) gp120 contains multiple neutralization epitopes (Ho et al. 1991; Fung et al. 1992; McKeating et al. 1993; Moore et al. 1993; Gorny et al. 1994; Warrier et al. 1994; Ditzel et al. 1995 and in prep.). The antibodies analyzed in these studies recognize a variety of epitopes, including linear, conformational, and glycan-dependent epitopes in the V2 domain. Whereas most of the anti-V2 monoclonal antibodies were relatively weak neutralizing antibodies, a chimpanzee monoclonal antibody, C108G, directed against a type-specific glycan-dependent epitope near the amino terminus of the V2 region, possessed very potent neutralizing activity for IIIB virus and related clones (Warrier et al. 1994; Wu et al. 1995). In another study, antibodies directed against a peptide in the V1 domain were isolated from a laboratory worker infected with HIV-1_{IIIB} and were shown to neutralize a related laboratory strain (Pincus et al. 1994).

Indirect data support a possible role for anti-V1/V2 antibodies in protection against transmission of HIV-1 and simian immunodeficiency virus (SIV). Selective transmission of HIV-1 variants from mothers to infants has been demonstrated (Wolinsky et al. 1992), and genetic variation in the 5′ portion of the *env* gene is not random in longitudinal samples isolated from seropositive mothers and their infected children but can be localized almost exclusively to the V1 and V2 loops (Lamers et al. 1993). For SIV, variation in SIV envelope sequences during progression to simian AIDS is primarily located in the V1 and V4 loops (Overbaugh et al. 1991), and the variation in V1 was associated with an increase of potential O-linked and N-linked glycosylation sites (Overbaugh and Rudensky 1992). These results suggest that immune selection is driving the variation observed in the V1 loop of HIV and SIV in vivo and are consistent with a protective effect of anti-V1/V2 antibodies in vivo.

Recent studies have shown that whereas laboratory-adapted T-cell-tropic (T-tropic) HIV-1 isolates are sensitive to neutralization by polyclonal patient sera and monoclonal antibodies directed against a number of different domains of the viral envelope proteins, primary macrophage-tropic (M-tropic) viruses and clinical isolates are much more difficult to neutralize (Golding et al. 1994; Matthews 1994; Moore et al. 1995). The following experiments were performed to address the basis of the relative sensitivities of T-tropic and M-tropic viruses to neutralization and to examine the ability of the V1/V2 domain to serve as a neutralization target for these viruses.

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host-cell factors. Performing the neutralization assay in a common batch of activated peripheral blood mononuclear cells (PBMCs) should also eliminate variability due to different target cells.

The two viruses used in these experiments contained related *env* genes with the identical V1/V2 domain. NL-HX contains an HXB2-derived *env* gene placed in an NL4-3 genetic background and has the T-tropic phenotype of the parental HXB2. NL-HX-ADA has a related structure to NL-HX, except that the central portion of the HXB2 *env* gene, encoding the V3-V5 portion of gp120, was replaced by the corresponding sequence of ADA, an M-tropic virus. This recombinant virus has the M-tropic phenotype of the ADA virus (Westervelt et al. 1992).

Differential Sensitivity of T-tropic and M-tropic Viruses to Monoclonal Antibodies to Neutralization Domains in gp120 and gp41

Neutralization assays were performed with SCD4 and with monoclonal antibodies directed against major sites on the HIV-1 Env proteins known to effectively mediate neutralization of laboratory strains of HIV-1. Chimpanzee MAb C108G is directed against a glycan-dependent epitope present in the V2 domain of HXB2 gp120 (Warrier et al. 1994); IgG-b12 is a human monoclonal antibody directed against a conserved conformational epitope that overlaps the CD4-binding site of gp120 (Burton et al. 1994); and 2F5 is a human monoclonal antibody directed against a conserved linear epitope in gp41 (Muster et al. 1993). IgG-b12 and 2F5 are considered to be among the most potent and broadly cross-reacting monoclonal antibodies described to date, and both possess reasonable neutralizing activity for a large fraction of primary viruses tested (Burton et al. 1994; Conley et al. 1994; Trkola et al. 1995).

All of the antibodies possessed potent neutralizing activity for the T-tropic virus. In the experiment illustrated, sCD4 and all of the monoclonal antibodies neutralized NL-HX with ND_{50} values in the range of 1–5 ng/ml (Fig. 2). However, when the same reagents were tested against the M-tropic virus, NL-HX-ADA, it was found that this virus was orders of magnitude less sensitive to neutralization by sCD4 and the antibodies directed against V3, CD4bs, and gp41 epitopes. In contrast to this, anti-V2 MAb C108G possessed potent neutralizing activity for both NL-HX and NL-HX-ADA. Whereas the ND_{50} values of C108G were only eightfold higher for NL-HX-ADA than for NL-HX, the differentials for the other reagents ranged from more than 100 to about 500 (Table 1). Two additional monoclonal antibodies directed against the V3 loop (C311E, Vijh-Warrier et al. 1996) and the CD4bs (S145A, Pinter et al. 1993) are included in Table 1, which also potently neutralize the T-tropic virus but have 1,500-fold lower activity for the M-tropic virus. The sensitive neutralization of the M-tropic virus by C108G suggested that its epitope, and perhaps the V2 domain in general, is a particularly sensitive neutralization target in M-tropic viruses.

Neutralizing Activity of the Anti-V1/V2 Antibody Fraction of a Human Serum for T-tropic and M-tropic Viruses

The V1/V2 domain has a complex folding pattern, and many of the epitopes in this region are conformational in nature (Wu et al. 1995). To develop a probe that could be used to characterize antibodies in human sera directed against native V1/V2 epitopes, we expressed the isolated V1/V2 domain of HXB2 gp120 as a hybrid glycoprotein in which the V1/V2 sequence was fused to the carboxyl terminus of a fragment of the murine leukemia virus Env protein, gp70 (Kayman et al. 1994). These fusion proteins

Table 1
Ratios of Neutralization Potencies of Monoclonal Antibodies for T-tropic (NL-HX) and M-tropic (NL-HX-ADA) Clones of HIV-1

MAb	*ND_{50}(NL-HX-ADA)/ND_{50}(NL-HX)*
C108G (αV2)	8
IgG-b12 (αCD4bs)	485
5145A (αCD4bs)	1600
C311E (αV3)	1500
rsCD4	109
2F5 (αgp41)	>380

Viral stocks were generated by transfection of molecularly cloned proviruses into 293 cells and assayed in PHA-activated PBMCs at input virus levels of 2 ng of p24 per milliliter. Viral infection was determined by a fluorescent focus assay at a time point when 2–5% of the control cultures were infected; NL-HX was assayed after 6 days of infection and NL-HX-ADA after 4 days of infection.

possess antibodies to a linear epitope in the V1 domain of the HXB2 gp120 sequence, which were reported to possess neutralizing activity for the NL4-3 virus (Pincus et al. 1994). Further analysis in our laboratory of the reactivity of this serum with the HXB2 V1/V2 fusion protein demonstrated the presence of antibodies directed against conserved conformational epitopes in the V1/V2 domain in addition to the antibodies to the type-specific linear V1 epitopes (A. Pinter et al., in prep.).

To determine whether the anti-V1/V2 antibodies in this serum contributed significantly to its neutralizing activity, an immunoabsorption experiment was performed in which the serum was passed over a Sepharose column that contained immobilized gp70-$V1/V2_{HXB2}$ fusion protein, and the bound antibodies were eluted with Tris-glycine buffer (pH 2.5) (Fig. 3). Absorption of this serum on the V1/V2 affinity column resulted in only a slight reduction in neutralization titer for the T-tropic virus but a considerable reduction in neutralization titer for the M-tropic virus (Table 2). This difference presumably reflects

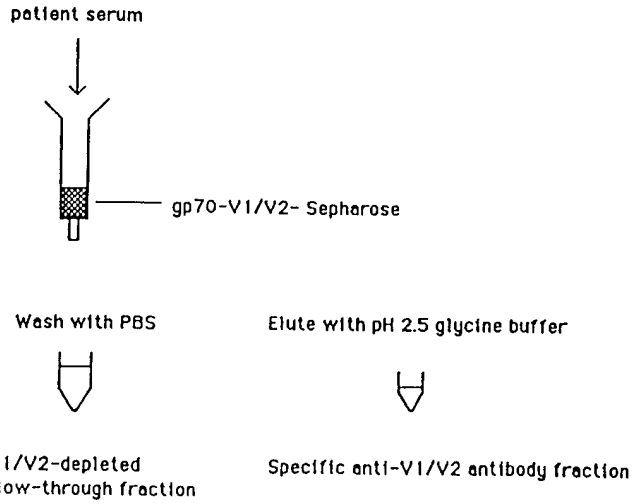

Figure 3
Fractionation of LWS serum on a V1/V2 immunoaffinity column.

viruses. This serum contained antibodies directed against both conserved conformational epitopes in the V1/V2 domain and type-specific linear epitopes in the V1 region. To test the role of these antibodies in HIV-1 neutralization, the anti-V1/V2 antibody fraction of this serum was isolated on an immunoaffinity column containing a recombinant fusion protein expressing the native HXB2 V1/V2 domain. The absorbed serum lost a significant fraction of its neutralizing activity for the M-tropic virus, and the V1/V2-specific antibodies eluted from the column possessed potent neutralizing activity for this virus, as well as lower neutralizing activity for the T-tropic viruses. These results demonstrate the ability of antibodies against epitopes in the V1/V2 domain to efficiently neutralize an M-tropic HIV-1 isolate and suggest that the V1/V2 region may be a particularly sensitive neutralization target in primary viruses.

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of 0.9 $\mu g\,ml^{-1}$ for the pH 2.5 fraction and 0.6 $\mu g\,ml^{-1}$ for the pH 1 fraction. ND_{90}s were not obtained for Ba-L, but the pH 1 fraction did have an ND_{80} endpoint of 1 $\mu g\,ml^{-1}$. For both viruses these activities were more potent than those obtained for most of the human monoclonal antibodies described above (*Figure 1* and *Table 1*), demonstrating that epitopes in the V1/V2 domain of macrophage-tropic gp120 were potent targets for neutralizing antibodies present in human serum.

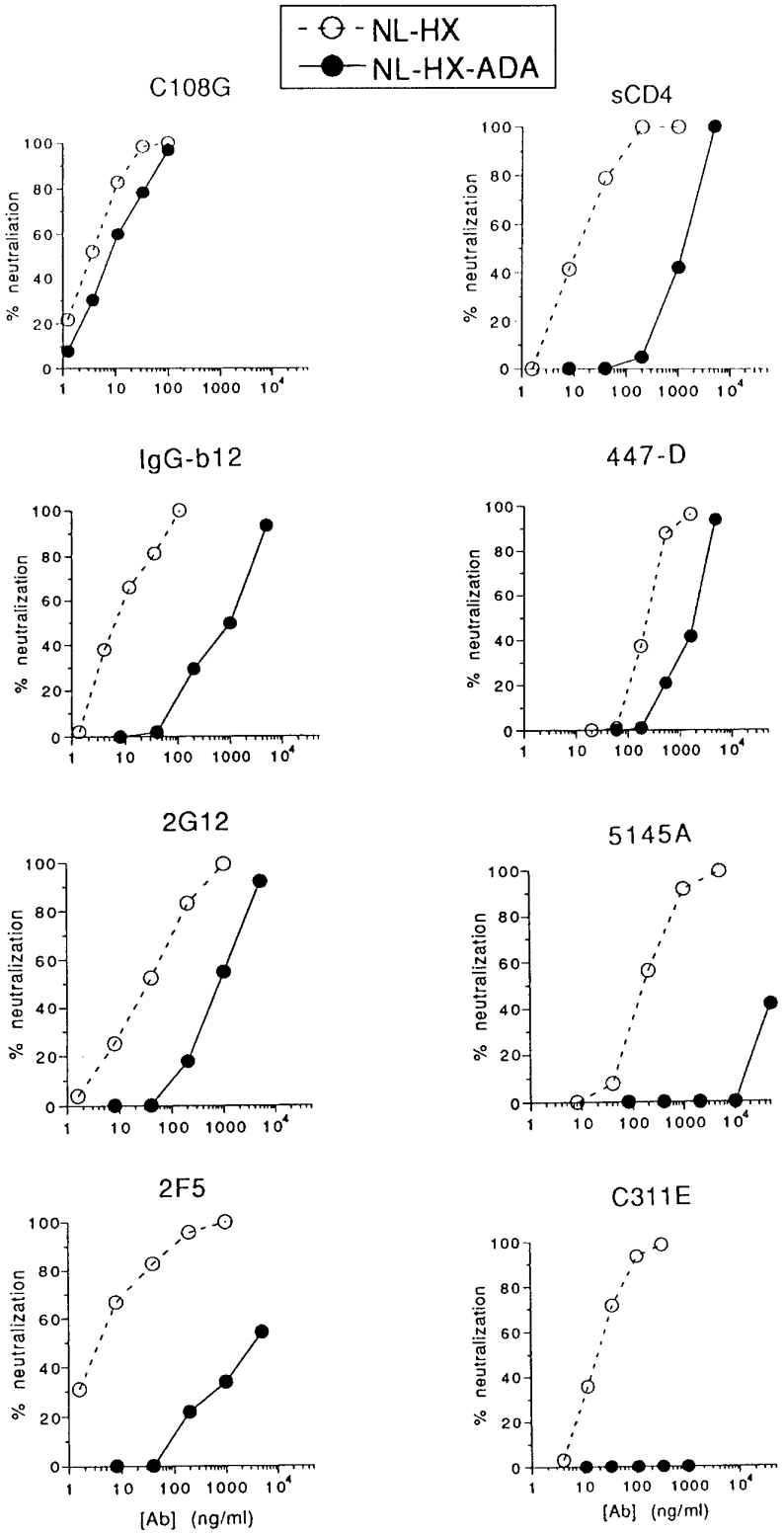

Figure 2 Titration curves for neutralization of T-tropic NL-HX and M-tropic NL-HX-ADA in PHA-activated human PBMCs by MAbs to different epitopes in gp120 and gp41. The abscissa represents concentration of MAbs in $\mu g/ml$

Table 1 50% and 90% endpoints (ng ml^{-1}) and endpoint ratios for neutralization of T-tropic (NL-HX) and M-tropic (NL-HX-ADA) clones of HIV-1

		ND_{50}			ND_{90}		
		NL-HX	NL-HX-ADA	NL-HX-ADA/ NL-HX	NL-HX	NL-HX-ADA	NL-HX-ADA/ NL-HX
C108G	(αV2)	2.5	7.6	3.0	20	66	3.3
2F5	(αgp41)	5.0	4060	812	130	—	—
IgG-b12	(αCD4-bd)	7.5	1000	133	71	4710	66.3
sCD4	(αCd4-bd)	14	1550	111	127	4250	33.5
C311E	(αV3)	21	>1000	>50	90	—	—
2G12	(αgp120)	38	900	24	560	4800	8.6
5145A	(αCD4-bd)	190	>50000	>263	950	—	—
447D	(αV3)	270	2125	8	840	4600	5.5

— indicates samples for which endpoints were not achieved

Characterization of antibodies produced upon immunization of rats with purified Case-A2 V1/V2 fusion protein

The potent neutralization of M-tropic viruses by the anti-V2 MAb, C108G, and the anti-V1/V2 antibodies isolated from human sera suggested that a vaccine capable of inducing such antibodies might provide protection against clinical isolates. Furthermore, the demonstration that neutralizing human antibodies were bound by the Case-A2 V1/V2 protein (*Figure 3*) indicated that this fusion protein expressed conserved epitopes capable of mediating neutralization of M-tropic HIV-1 isolates. We were therefore interested in determining whether immunization with the Case-A2 V1/V2 fusion protein would induce antibodies capable of neutralizing primary isolates. To test this, we immunized a number of Fischer rats with the purified V1/V2 protein. These animals received an initial dose of 5 μg kg^{-1} of the immunogen in MPL/TDM adjuvant (RAS, from Ribi Immunochem Research Hamilton, Mo) followed by monthly boosts with 1 μg kg^{-1} of the antigen in the same adjuvant. This resulted in the production of crossreactive antibodies that recognized a number of different recombinant V1/V2 fusion proteins and recombinant gp120s; the reactive gp120s included representatives of both T-tropic isolates (LAV and SF2), and M-tropic isolates (Ba-L and JR-FL), and the reactive V1/V2 fusion proteins included distant sequences from clade D and clade E isolates, data not shown. This shows that the Case-A2 V1/V2 protein expressed epitopes that were present in heterologous gp120s and conserved across clades.

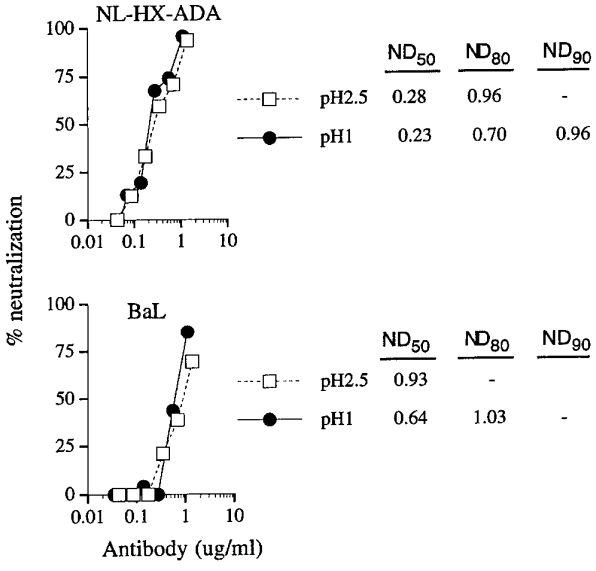

Figure 3 Neutralization of two macrophage-tropic HIV-1 isolates, NL-HX-ADA and Ba-L, by V1/V2-specific antibody fractions of one HIV-positive human serum, isolated on a Case-A2 V1/V2 affinity column

In preliminary experiments, these immune sera were found to possess neutralizing activity for a number of primary HIV-1 isolates. To remove nonspecific neutralizing activities commonly found in normal rat sera and to allow accurate quantitation of the V1/V2-specific antibodies in these sera, antibodies were isolated from the serum of one animal by immunoaffinity chromatography. Since activity against the V1/V2 fusion protein present in the rat immune serum consisted of antibodies directed against both the gp70-derived carrier sequence and the V1/V2 domain, the anti-gp70 antibodies were first absorbed on an affinity column containing the gp70-derived portion of the V1/V2 fusion protein, and then antibodies specific for the V1/V2 region were fractionated on a column containing the complete Case-A2 V1/V2 fusion protein. Specifically absorbed antibodies were recovered from each column by elution with low pH buffers, quantified by ELISA, and tested for neutralizing activity against the recombinant M-tropic isolate, NL-HX-ADA, and against a clade B clinical isolate, 92US716B (*Figure 4*).

Greater than 95% of specific antibodies to gp70 and V1/V2 fusion protein were depleted after passage of serum over the respective columns (data not shown), and a fraction of these activities was recovered in the pH 2.5 and pH 1 eluates. The antibodies eluted from the gp70 column had no detectable neutralizing activity when tested against NL-HX-ADA (*Figure 4*A). The gp70-depleted serum had an ND_{50} against NL-HX-ADA of 19 μg ml^{-1} of rat IgG, similar to that of the original serum and corresponding to a serum dilution of approximately 1:250, and an ND_{50} for 92US716B of approximately 100 μg ml^{-1}, corresponding to a serum dilution of approximately 1:50. These neutralizing activities were absent from the V1/V2 column flow-through (*Figure 4*B and *4*C), indicating that they were due to V1/V2-specific antibodies. In contrast to the antibodies eluted from the gp70 column, the pH 2.5 and pH 1 fractions of the V1/V2 column possessed

significant neutralizing activity for both viruses. Approximately 5% of the total immunoglobulin applied to the V1/V2 column was recovered in the two acid eluates; the pH 2.5 fraction had a rat IgG concentration of 194 $\mu g\,ml^{-1}$, while the pH 1 fraction had a rat IgG concentration of 82 $\mu g\,ml^{-1}$. The pH 1 fraction had the higher neutralizing activity, with ND_{50}s of 0.68 $\mu g\,ml^{-1}$ for NL-HX-ADA and 0.84 for 92US716B, while the pH 2.5 eluates had ND_{50}s of 3.8 $\mu g\,ml^{-1}$ for NL-HX-ADA and 6.1 $\mu g\,ml^{-1}$ for 92US716B. The neutralizing activity of the V1/V2-specific antibodies present in the pH 1 fraction for NL-HX-ADA was more potent than that of any of the monoclonal antibodies described in *Figure 1* and *Table 1*, other than the V2-specific antibody C108G. These results further demonstrate the sensitive neutralization of macrophage-tropic and primary isolates by antibodies against epitopes in the V1/V2 domain, and indicate the potential of the Case-A2 V1/V2 fusion protein for eliciting such neutralizing antibodies.

DISCUSSION

Progress towards an effective HIV vaccine has been hindered by the inability of current vaccine candidates to elicit production of antibodies capable of neutralizing primary viruses[1,2,48,49]. The refractivity of primary viruses to vaccinee sera is presumably related to their general resistance to neutralization by sCD4[47,50] and monoclonal antibodies[3,4]. The reason for the difference in sensitivity of primary viruses and laboratory isolates is not clear; it has been suggested that epigenetic factors introduced by the cells used to prepare the virus, such as differential glycosylation or incorporation of host cell adhesion proteins into virion membranes, may be factors[51–53]. The data in this paper showing that MAbs to most of the previously described neutralization domains exhibit very large differences in neutralizing activities for a matched pair of molecularly cloned T cell-tropic and macrophage-tropic viruses that were prepared in the same cells and assayed in a common batch of activated PBMC indicate that this phenomenon is determined genetically by the sequence and structure of the viral envelope protein. These results also show that this resistance is epitope-specific rather than absolute, since at least one antibody to a V2 epitope has potent neutralizing activity for both the T-tropic and M-tropic viruses the T-tropic virus.

The MAbs compared in *Figure 1* and *Table 1* include antibodies IgG-b12, 2F5, 447-D and 2G12, which have all been described as possessing potent neutralizing activities for primary viruses[17–19,39]. The potency of these antibodies for the M-tropic NL-HX-ADA was lower than for the T-tropic NL-HX by factors ranging from 8 to over 800, as measured by ND_{50} ratios, indicating that these antibodies preferentially neutralized T-tropic viruses. It thus appears that the neutralizing activity against primary isolates reported for these antibodies reflects their overall high potencies, rather than any particular characteristic of the epitopes recognized by the MAbs. The relative resistance of NL-HX-ADA to neutralization by these MAbs could not be accounted for simply by direct structural changes in the epitopes expressed in the two viruses, since these effects were seen for antibodies against strongly conserved epitopes in the CD4-binding site of gp120 and for the 2F5 epitope in gp41, which was identical for the two viruses.

On the other hand, certain epitopes in the V1/V2 domain may be preferential neutralization targets for macrophage-tropic viruses. This is suggested by the potent neutralization of NL-HX-ADA by the anti-V2 MAb C108G, and the effective neutralization of this virus and the primary isolates Ba-L and 92US716B by the V1/V2-specific antibodies isolated from a naturally infected human serum and from a rat immunized with a recombinant V1/V2 fusion protein. Neutralization potencies similar to or greater than those described in this report have been obtained for additional infected human and immunized rat sera fractionated in the same way, and thus the results reported in this study appear to be generalizable. The ability to efficiently neutralize primary viruses is not, however, a general property of anti-V1/V2 antibodies, since a number of rodent MAbs with varying neutralizing activities for IIIB virus[26,27] did not have detectable neutralizing activity for NL-HX-ADA (data not shown), nor did we detect significant neutralization of NL-HX, NL-HX-ADA or Ba-L by 697-D, a human MAb directed against a conserved conformational V1/V2 epitope[32]. Further characterization of the epitopes recognized by both neutralizing and non-neutralizing antibodies to this region is needed to understand the basis for these distinctions.

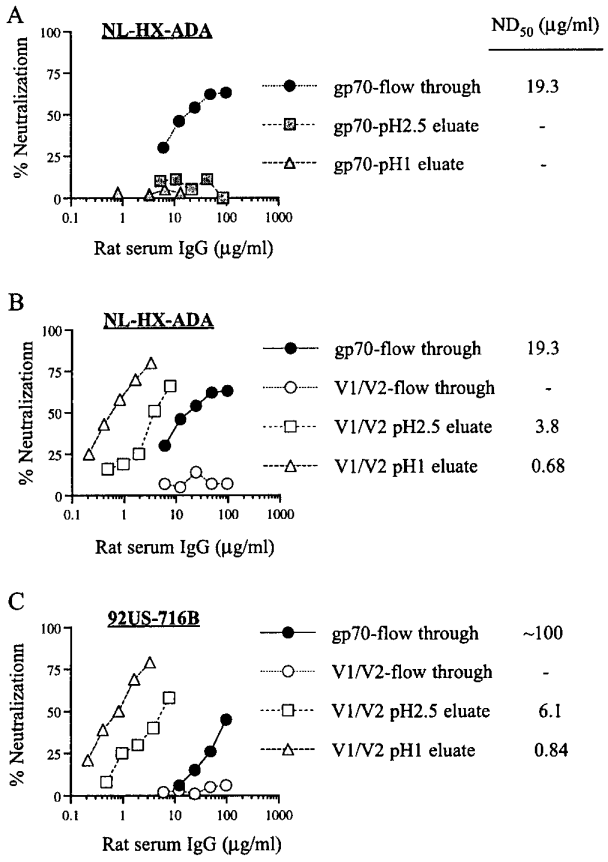

Figure 4 Neutralization of NL-HX-ADA (A and B) and a clinical isolate, 92-US716B (C), by antibodies induced by immunizing a rat with the purified Case-A2 V1/V2 fusion protein

There is currently a widespread awareness of the need to determine the immunological correlates of protection against HIV-1, and in particular, to define the specificity of antibodies in the sera of certain patients that neutralize primary viruses grown in peripheral blood mononuclear cells[54]. The results described in this study suggest that antibodies to conserved epitopes in the V1/V2 domain of gp120 contribute to such neutralization and thus may be a correlate of immunity against HIV. These results further suggest that a recombinant immunogen related to the V1/V2 fusion protein described in this paper may be an effective reagent for eliciting such protective antibodies. Additional vaccination studies with this immunogen are currently being carried out in a primate model, to determine whether these animals produce similar neutralizing responses. These studies will provide insight into the ability of a subunit vaccine based on the V1/V2 domain to induce crossreactive neutralizing antibody responses that are effective against a broad range of HIV-1 clinical isolates.

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Efficient induction of protective anti-malaria immunity by recombinant adenovirus

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The immunogenicity of a previously constructed replication-defective recombinant adenovirus expressing the CS protein of Plasmodium yoelii *was compared with that of irradiated sporozoites. We found that immunization of BALB/c mice with a single dose of this recombinant adenovirus induced a much greater CS-specific T-cell response compared with immunization with irradiated sporozoites. More importantly, we found that this recombinant adenovirus induces similar or higher levels of protective immunity than those induced by irradiated sporozoites, eliciting an appreciable resistance to malaria infection. © 1998 Elsevier Science Ltd. All rights reserved*

Keywords: malaria; adenovirus; protection

INTRODUCTION

The finding that radiation-attenuated sporozoites can induce complete protection against malaria infection, not only in experimental animals but also in humans[1],demonstrated for the first time the feasibility of effective vaccination against this disease. This success has encouraged research aimed at developing a sub-unit vaccine, since the approach used to immunize humans, namely, repeated exposure to the bites of numerous malaria-infected, irradiated mosquitoes, is not practical and is only feasible on a very small experimental scale.

Immunization with irradiated sporozoites also induces T-cell effector mechanisms, which appear to be primarily mediated by CD8+ cytotoxic T cells which inhibit the development of the liver stage parasites. This was shown for two rodent malaria species, *Plasmodium berghei* and *P. yoelii*, in which the *in vivo* depletion of CD8+ T cells abolished sterile immunity induced by sporozoites, as determined by the occurrence of blood infection[2,3]. The protective role of CD8+ T cells was further confirmed by the experiments in which the adoptive transfer to naive mice of CD8+ T-cell clones specific for CS or SSP2, another pre-erythrocytic antigen, conferred protection against sporozoite challenge[4–6]. Finally, CD4+ T cells were also shown to play a role in protective immunity against the pre-erythrocytic stages of malaria parasites. CD4+ T-cell clones, specific for the CS protein, and for another, as yet unidentified antigen, shared by sporozoites and blood stage parasites, were shown to confer protection against challenge with *P. yoelii* and *P. berghei* sporozoites[7–9].

In recent years, a new approach to the development of vaccines has become available, which is based on the use of microbial organisms, engineered to express foreign genes as vectors for antigen delivery. These vectors offer many advantages, such as the safety of non-pathogenic vectors[10], and, more importantly, the potential to elicit not only a humoral response, but also cell-mediated response[10]. Since it has been demonstrated that malaria-specific CD8+ T cells have a major role in immunity targeted against the parasite's liver stages, several attempts have been made to induce protective anti-plasmodial CD8+ T cells *in vivo*, using various recombinant viral and bacterial vectors[11–13]. In most of these instances, the protection obtained against the highly infective rodent malaria parasite *P. yoelii* was partial and could only be achieved by immunizing in succession with two different recombinant vectors, both expressing the same malaria antigen[13].

Our decision to use the adenovirus system was based on several attractive features that the adenovirus possesses with regard to the development of a sub-unit vaccine. First, the virion is relatively stable, and thus the inserted foreign genes do not tend to mutate after successive rounds of viral replication. Secondly, the adenoviral genome has been extensively investigated for many years, and the complete DNA sequence of several serotypes is known. This greatly facilitates the ease which the adenoviral genome can be manipulated by recombinant DNA techniques[14]. Thirdly, these viruses efficiently transfer genes to both replicating and non-replicating cells, and the transferred genetic information remains epi-chromosomal, thereby avoiding

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The Hypervariable Domain of the Murine Leukemia Virus Surface Protein Tolerates Large Insertions and Deletions, Enabling Development of a Retroviral Particle Display System

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The surface proteins (SU) of murine type-C retroviruses have a central hypervariable domain devoid of cysteine and rich in proline. This 41-amino-acid region of Friend ecotropic murine leukemia virus SU was shown to be highly tolerant of insertions and deletions. Viruses in which either the N-terminal 30 amino acids or the C-terminal 22 amino acids of this region were replaced by the 7-amino-acid sequence ASAVAGA were fully infectious. Insertions of this 7-amino-acid sequence at the N terminus, center, and the C terminus of the hypervariable domain had little effect on envelope protein (Env) function, while this insertion at a position 10 amino acids following the N terminus partially destabilized the association between the SU and transmembrane subunits of Env. Large, complex domains (either a 252-amino-acid single-chain antibody binding domain [scFv] or a 96-amino-acid V1/V2 domain of HIV-1 SU containing eight N-linked glycosylation sites and two disulfides) did not interfere with Env function when inserted in the center or C-terminal portions of the hypervariable domain. The scFv domain inserted into the C-terminal region of the hypervariable domain was shown to mediate binding of antigen to viral particles, demonstrating that it folded into the active conformation and was displayed on the surface of the virion. Both positive and negative enrichment of virions expressing the V1/V2 sequence were achieved by using a monoclonal antibody specific for a conformational epitope presented by the inserted sequence. These results indicated that the hypervariable domain of Friend ecotropic SU does not contain any specific sequence or structure that is essential for Env function and demonstrated that insertions into this domain can be used to extend particle display methodologies to complex protein domains that require expression in eukaryotic cells for glycosylation and proper folding.

The external proteins of enveloped viruses mediate binding to and penetration of the host cell. Retroviral envelope proteins (Env) consist of a peripheral, receptor-binding surface protein (SU) subunit and a membrane-spanning transmembrane protein (TM) subunit that contains an N-terminal fusion domain. They are synthesized as a single polypeptide that is proteolytically processed into the mature Env complex (31). In the type-C murine leukemia virus (MuLV) and related viruses, the N- and C-terminal sequences of SU are independent globular domains (20, 35), with receptor-binding activity residing in the N-terminal domain (2–4, 10, 25, 29). The recently determined crystal structure of the receptor-binding N-terminal domain of an ecotropic MuLV SU suggests that a conserved core of β sheets in an immunoglobulin fold provides the structural underpinning for presenting the receptor-binding site assembled from sequences that vary among receptor classes (7). Many of these Envs contain a labile disulfide bond between SU and TM (17, 23, 28, 32–35, 52) that involves a pair of cysteines present in a highly conserved structural motif near the beginning of the C-terminal domain of SU and that may be important in Env function (39). Connecting the N- and C-terminal globular domains of SU is a region that is rich in proline. This proline-rich region can be divided into two domains by sequence comparisons: an N-terminal domain of 12 residues that is highly conserved among MuLV SUs and somewhat conserved among a broader group of viruses and a C-terminal domain that is hypervariable. Deletion of the conserved proline-rich domain results in failure of processed Env complex to be incorporated into virions, while the hypervariable domain tolerates significant deletions and small insertions, some of which weaken the association between SU and TM (53).

In this report, the function of the hypervariable domain linking the N-terminal receptor-binding domain and the highly conserved C-terminal domain of MuLV SUs was further investigated by constructing a series of small and large insertions and deletions in this region of Friend ecotropic MuLV (Fr-MuLV). Insertions into the N-terminal portion of the hypervariable domain destabilized the interaction between SU and TM, sometimes sufficiently to interfere with viral growth. In contrast, the C-terminal portion of the hypervariable domain was found to be extremely tolerant of modification, including the insertion of large sequences containing N-linked glycosylation sites and internal disulfide bonds. These modified Envs retained full function, and the inserted sequences were exposed at the surface of viral particles, where they were efficiently recognized by antibodies and other ligands directed against the inserted sequences. Furthermore, it was demonstrated that virions carrying such insertions could be physically selected out of mixed populations, thus enabling a novel retroviral particle display system suitable for eukaryotic sequences that cannot be expressed in bacterial systems. Similar insertions may also prove to have relevance for redirecting the cell specificity of the virus, allowing targeting of retroviral gene therapy delivery to cells of choice.

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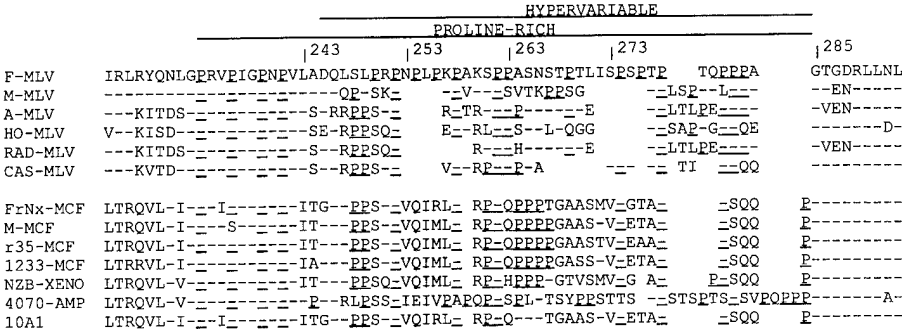

FIG. 1. Sequence conservation near the proline-rich domain of MuLV SUs. Residues matching that of the Fr-MuLV sequence are indicated with a hyphen; Pro residues are underlined; gaps introduced for alignment have been left blank. The first group of sequences are from ecotropic *envs*; the second group are from *envs* of other receptor classes. F-MLV (16); M-MLV (45); A-MLV (18); HO-MLV (49); RAD-MLV (22); CAS-MLV (42); FrNx-MCF (1); M-MCF (5); r35-MCF (41); 1233-MCF (46); NZB-XENO (27); 4070-AMP and 10A1 (30).

MATERIALS AND METHODS

Viruses and cell lines. The MuLV *env* was from clone 57 Fr-MuLV (26). MuLV was expressed from a chimeric Fr-MuLV 2 long terminal repeat colinear genomic plasmid (pLRB303 for wild-type virus) containing most non-*env* sequences from the FB29 clone (15). Mouse NIH 3T3 fibroblasts were maintained as previously described (14). SEC-CHO, a CHO cell line that secretes a truncated, soluble form of the HIV_{HXB2} Env precursor, gp140, and its cleavage product, gp120, was obtained from Judith White and maintained as described previously (51). Mutant viruses were expressed by transfecting the genomic viral plasmids into 3T3 cells by using Lipofectamine (GibcoBRL). Insertion mutations, introducing *Nhe*I, *Eco*47III, *Ngo*MI, and *Nar*I restriction sites and encoding a 7-amino-acid sequence, ASAVAGA (5′-GCT AGC GCT GTT GCC GGC GCC-3′), were constructed at each of the sites indicated in Fig. 1 by PCR overlap mutagenesis (11). Human monoclonal antibody (MAb) 5145a recognizes a CD4 binding site epitope on human immunodeficiency virus type 1 (HIV-1) SU (gp120) (38). A 252-amino-acid 5145a scFv gene fragment with a $(Gly_4Ser)_3$ sequence linking the heavy- and light-chain variable domains (12) was constructed by PCR overlap mutagenesis from clones provided by Ellen Murphy and cloned into various insertion site plasmids on *Nhe*I and *Ngo*MI ends, retaining the AS dipeptide N-terminal to the scFv domain and the AGA tripeptide C-terminal to it. The 96-amino-acid gp120 V1V2 domain of the CaseA2 HIV-1 sequence, which has been described previously (37), was inserted between residues 273 and 274 by using *Nhe*I and *Nar*I restriction sites, retaining the AS dipeptide N-terminal to the V1V2 domain and the GA dipeptide C-terminal to it.

Immunoassays. Goat anti-Rauscher gp70 serum and goat anti-Rauscher p30 serum were obtained from Quality Biotech (Camden, N.J.). Rat MAb 10BA10 specific for Fr-MuLV $p12^{gag}$ (14) and mouse MAb SC258, provided by Abbott Laboratories and specific for a conformational epitope in the V1V2 domain of HIV-1 gp120 (24, 54), have been previously described. Viral infection was detected by immunofluorescence assay (IFA) by using 10BA10 as previously described (14). Following transfection with a plasmid expressing a noninfectious virus, no increase in Gag^+ cells is seen by IFA beyond 18 h posttransfection, indicating that all successfully transfected cells express detectable Gag by this time point (14). Specific infectivity was examined by determining the percent of cells producing $p12^{gag}$ 18 h following a standard infection protocol by using serial dilutions of virus containing culture supernatants with similar amounts of $p30^{gag}$. The most-concentrated sample was a 1:20 dilution of culture supernatant. Viral proteins were characterized by radioimmunoprecipitation (RIP) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography as previously described (36). Radioisotopes were obtained from New England Nuclear.

Enrichment procedures. Pansorbin cells (Calbiochem), prepared for RIP, were washed five times with 10 volumes of PBS and then stored at 4°C as a 10% suspension. His_6-tagged protein A was prepared as described previously (40) from the expression plasmid kindly provided by Tim Hunt of the Imperial Cancer Research Fund. Ni^{2+}-nitrilotriacetic acid (NTA) agarose (Qiagen) was washed three times in PBS and resuspended to a 50% slurry in PBS. One-half volume of His_6 protein A at 1.5 mg/ml was added to the washed Ni^{2+}-NTA agarose slurry, followed by the addition of 3 volumes of PBS and overnight incubation at 4°C. Culture supernatants containing either wild-type or V1V2-SU virus were mixed in proportions to give either an excess of wild-type virus for positive enrichment experiments or an excess of V1V2-SU virus for negative enrichment experiments. A 0.5-ml portion of the virus mixture was incubated with MAb SC258 at 37°C for 1 h. For positive enrichment, the virus mixture was used to suspend 0.05 ml of packed Ni^{2+}-NTA agarose with prebound His_6 protein A and rotated at room temperature for 1 h. The Ni^{2+}-NTA agarose was washed twice with 0.5 ml of PBS by pelleting and then suspended in 0.2 ml of 10 mM EDTA in PBS for 5 min at room temperature. The Ni^{2+}-NTA agarose was removed by centrifugation, and 0.2 ml of 40 mM $MgCl_2$ was added immediately. For negative enrichment, the virus mixture was used to suspend 0.01 ml of packed Pansorbin and rotated at room temperature for 1 h. Pansorbin was then removed by centrifugation. Aliquots of unseparated virus mixtures (starting materials), Pansorbin supernatants (negatively enriched sample), and Ni^{2+}-NTA agarose eluates (positively enriched samples) were used to infect 3T3 cells, and virus growth was monitored by IFA. When the cultures were fully infected, [^{35}S]cysteine-labeled culture supernatants were prepared and analyzed by RIP with goat anti-gp70 serum followed by SDS-PAGE and autoradiography. The amount of each SU was quantitated on a Molecular Dynamics PhosphorImager.

RESULTS

The hypervariable domain of Fr-MuLV SU is tolerant of insertions and deletions. Comparison of the proline-rich central domains of murine type-C retroviral envelope genes (*env*) indicates that the first four of these prolines constitute a motif conserved among these *envs*, while the following region (residues 244 to 284 in Fr-MuLV) is hypervariable even within receptor classes (Fig. 1). To examine the tolerance of the hypervariable domain to modification, a 7-amino-acid insert, ASAVAGA, a sequence expected to have little intrinsic structure, was placed at five sites across this region. Mutants in which the ASAVAGA sequence replaced residues 244 to 273 or 264 to 285 were also constructed.

All of the ASAVAGA insertion and substitution mutants grew normally. Growth curves following transfection of plasmids expressing selected mutant viruses are presented in Fig. 2A. Differences of 1 day or less in initial growth were attributable to small differences in transfection efficiency. The specific infectivities of the virus present at the end of these growth curves were also similar to that of wild type (Fig. 2B). Despite these normal growth characteristics, examination of the envelope proteins associated with virus particles revealed that insertion of ASAVAGA following residue 253 significantly destabilized the interaction between SU and TM (Fig. 3). Cultures resulting from the above-mentioned transfections were labeled with [^{35}S]cysteine, and particle-associated proteins were separated from soluble proteins in culture media by pelleting virus. In each case, essentially all of the core protein, $p30^{gag}$, was found in the viral pellet (data not shown). The majority of wild-type SU was associated with the viral pellet. This was also the case for all of the ASAVAGA mutants except the 253/254 insertion, for which most of the SU was soluble protein found in the supernatant fraction (Fig. 3A). Interestingly, particle association of the 243/274 ASAVAGA substitution mutant SU was close to normal, despite deletion of the 253/254 region that was sensitive to insertion.

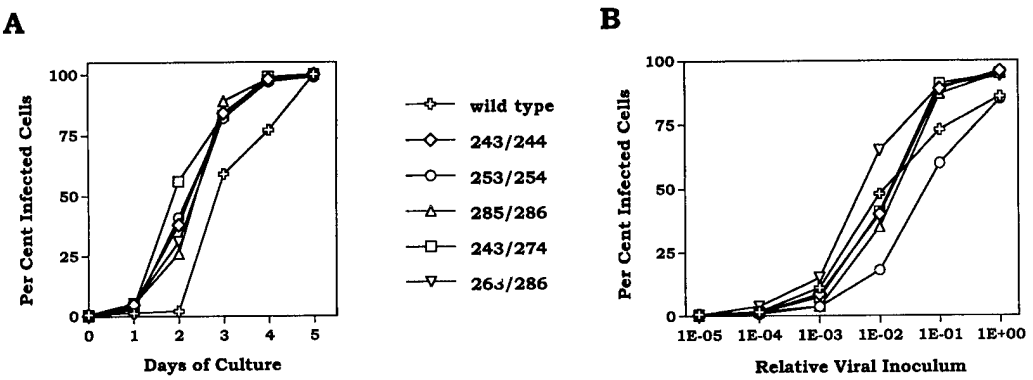

FIG. 2. Growth characteristics of ASAVAGA insert mutants. (A) 3T3 cells were transfected with the expression plasmid for the indicated viruses, and slides were prepared for IFA daily until viral infection reached 100%. Day 0 represents data from 18 h posttransfection, at which time between 0.1 and 0.7% of cells were expressing Gag. (B) Serial dilutions of culture supernatants from the ends of the growth curves in panel A were infected into 3T3 cells, and the percentage of infected cells was determined 18 h later by IFA.

To further explore the degree of tolerance for insertions within the hypervariable domain, large insertions consisting of a single-chain antibody binding domain (scFv), derived from human MAb 5145a that recognizes a CD4 binding site epitope on the HIV-1 SU (gp120), were constructed. As shown in Fig. 4A, insertion of the scFv domain was well tolerated at the 273 and 285 insertion sites but not at the 243 and 253 insertion sites, where a significant growth delay resulted. The growth defect of the 243/244 and 253/254 scFv insertion mutants correlated with severe decreases in the specific infectivity of viral particles (Fig. 4B). The low specific infectivity of these virions indicated that the virus present at the end of these growth curves was mutant, despite the rapid spread of infection following the 4-day lag. The apparent discrepancy between this eventual rapid spread (Fig. 4A) and the extremely low specific infectivity of the virions (Fig. 4B) may reflect a contribution of cell-to-cell infection to viral spread in culture and/or the additional opportunity for shedding of SU afforded by the handling of viral supernatants in the specific infectivity experiment. These growth defects were consistent with the greatly reduced amounts of particle-associated SU found for the 243/244 and 253/254 scFv insertion mutants (Fig. 5). A small decrease in the amount of particle-associated SU was also seen for the scFv 285/286 insertion mutant, at the C-terminal boundary of the hypervariable domain (Fig. 5). There was a small amount of material in the supernatants of the scFv insert viruses that migrated similarly to wild-type SU. It appeared to be a C-terminal fragment of the mutant SU, since in each case its degree of particle association matched that of the intact scFv SU. Although the scFv insertion was not tested following residue 263, other large insertions (such as V1V2 and V4C4 domains of HIV-1 SU) at this site did not affect virus growth (data not shown). Taken together, these data show that the hypervariable domain of MuLV SU is highly tolerant of insertion and deletion, particularly in its central region.

Foreign sequences inserted within the hypervariable domain express active conformational structures that are exposed on the virus particle. To determine whether the 5145a scFv domain inserted into the hypervariable domain of SU folded properly and was exposed on the surface of the virus particle, the ability of soluble and particle-associated SUs containing this insert to bind antigen was investigated. SEC-CHO, a CHO cell line that expresses a form of HIV-1 Env that is truncated at the boundary between the ectodomain of TM and its transmembrane domain, was used as the source of antigen. These cells secrete both the primary translation product, gp140, and gp120, the product of cleavage at the normal site between SU and TM (51). Culture supernatant of SEC-CHO labeled with [^{35}S]cysteine was mixed with culture supernatant of MuLV-producing 3T3 cells also labeled with [^{35}S]cysteine, particle-associated and soluble proteins were separated by centrifugation, and samples were immunoprecipitated with serum specific for MuLV SU (gp70) or for HIV-1 SU (gp120) (Fig. 6). In the wild-type control, MuLV SU was precipitated from both particulate and soluble fractions by using the anti-gp70 serum, while HIV-1 SU was precipitated only from the soluble fraction and only with the anti-gp120 antiserum. These results demonstrated that HIV-1 SU does not associate with wild-type MuLV SU or with any other component on the surface of MuLV particles. For the 273/274 scFv insertion mutant, the distribution of MuLV SU between particulate and soluble fractions, detected by immunoprecipitation with the anti-gp70 serum, was similar to that of wild type, as expected. However, unlike the results for wild-type virus, HIV-1 SU was detected in the particulate fraction containing the 273/274 scFv insert virions by immunoprecipitation with anti-gp120 serum. This association of HIV-1 SU with the mutant virus was dependent on the association of the scFv insert SU with virions, since it was not seen for the 253/254 scFv insertion mutant that contained only a trace of MuLV SU in the particulate fraction due to its defect in SU-TM interaction. Consistent with these data, a large portion of the HIV-1 SU was coprecipitated with the MuLV SU by the anti-gp70 serum from all fractions containing both scFv insert SU and HIV-1 SU. In contrast, coprecipitation of MuLV SU with HIV-1 SU with the anti-gp120 serum was not detected in any sample, presumably reflecting higher specific radioactivity and lower concentration for the HIV-1 SU

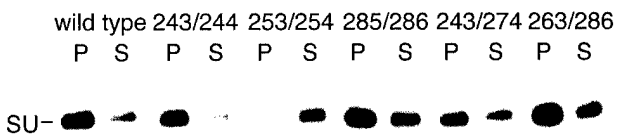

FIG. 3. Particle association of ASAVAGA insert SUs. 3T3 cells producing the indicated viruses were labeled with [^{35}S]cysteine, and culture supernatants were separated into soluble (S) and particulate (P) fractions by sedimentation. Samples were analyzed by RIP with hyperimmune anti-gp70 serum, followed by SDS-PAGE and autoradiography.

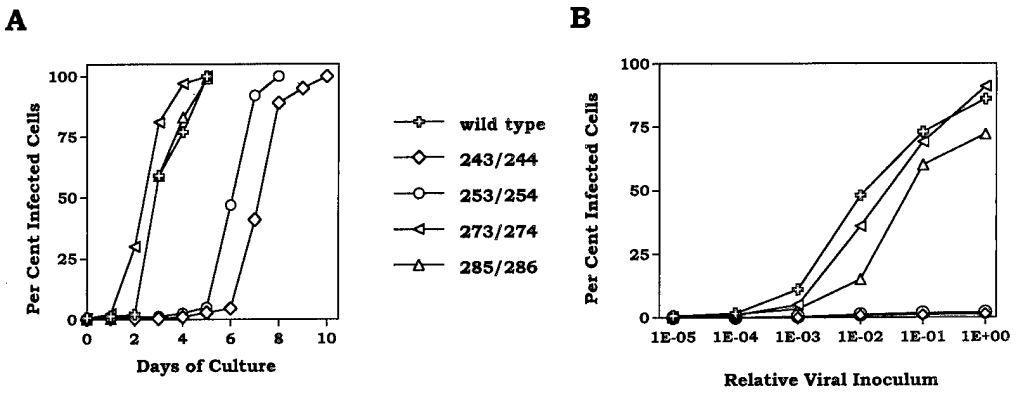

FIG. 4. Growth characteristics of 5145A scFv insert mutants. (A) 3T3 cells were transfected with the expression plasmid for the indicated viruses, and slides were prepared for IFA daily until viral infection reached 100%. Day 0 represents data from 18 h posttransfection, at which time between 0.1 and 0.7% of cells were expressing Gag. (B) Serial dilutions of culture supernatants from the ends of the growth curves in panel A were infected into 3T3 cells, and the percentage of infected cells was determined 18 h later by IFA.

than for the MuLV SU. These data clearly indicated that the 5145a scFv expressed within the hypervariable domain of MuLV SU efficiently bound antigen both on the surface of intact virions and free in solution.

Foreign sequences inserted within the hypervariable domain provide the basis for a retroviral particle display system. The efficient expression of inserted sequences on the surface of intact retroviral particles suggested the possibility of using such inserts for a retroviral display system. MuLV expressing the V1/V2 domain of HIV-1 gp120 as a 273/274 insert was used to demonstrate that particles expressing inserted sequences could be separated based on the binding activities of the inserts. The V1/V2 domain used consists of 96 amino acids and contains two disulfide bonds and eight signals for N-linked glycosylation (50). It presents a number of linear and conformational epitopes recognized by available MAbs (data not shown). The wild-type SU and V1/V2-bearing SU are easily resolved by SDS-PAGE due to a difference of about 30 kDa in apparent molecular size, allowing quantitation of the ratio of the two viruses present before and after separation.

Methods for selectively depleting (negative enrichment) or recovering (positive enrichment) V1/V2-expressing particles from mixtures with wild-type particles using anti-V1/V2 MAbs were established. In a negative enrichment, the desired viruses are those that are not bound by a specific antibody. This was achieved by removing virus particles bound to MAb SC258, specific for a conformational epitope expressed on the V1/V2 insert, on standard Pansorbin cells. A mixture of wild-type and V1/V2-chimeric virus was incubated with SC258 and then with Pansorbin, and unbound viruses were recovered following centrifugation. The initial virus mixture and the virus recovered after separation were amplified by infection into 3T3 cells, and [^{35}S]cysteine-labeled supernatants were analyzed by immunoprecipitation with anti-gp70 serum (Fig. 7A). The ratio between V1/V2 SU and wild-type SU in the starting mixture was 5.3:1 after amplification, while after depletion and amplification it was 1:77, overall a 410-fold enrichment for the nonreactive virus or depletion of the reactive virus. The epitope seen by SC258 requires correct glycosylation and disulfide-bond formation of the V1/V2 domain (54). Thus, the successful depletion of V1/V2 SU virus with SC258 demonstrated that, like scFv domains, the V1/V2 domain is both correctly folded and exposed on the surface of virus particles when inserted into the hypervariable domain of MuLV SU.

Positive enrichment requires recovery of infectious virus from the bound state. Standard conditions used to disrupt antibody-antigen complexes, such as extremes of pH or high concentrations of chaotropic agents, are lethal to MuLV (data not shown). To overcome this problem, a recombinant protein A containing a six-histidine affinity tag (40) was used. This provided a system in which the binding of antibody to a solid support, Ni^{2+}-NTA resin, was reversible under mild conditions. Viruses complexed with MAb were adsorbed on Ni^{2+}-NTA resin carrying His_6-protein A, washed, and eluted with 10 mM EDTA. Recovered viruses were amplified by infection into 3T3 cells following immediate addition of $MgCl_2$, and the ratios of V1/V2 SU to wild-type SU in labeled supernatants from mixtures before and after separation were compared (Fig. 7B). The ratio of chimeric to wild-type SU increased from 1:2.7 to 12:1, overall a 32-fold enrichment for reactive virus when SC258 was used at 20 μg/ml. Similar results were obtained with as little as 1 μg of SC258 per ml.

DISCUSSION

A central proline-rich and hypervariable domain is a conserved structural feature of all classes of MuLV Env (30). This study demonstrates that a large fraction of this hypervariable domain in the Fr-MuLV SU (at least the N-terminal three-fourths and the C-terminal one-half) can be deleted without significant effect on Env function and that inserts containing either 252 amino acids or 96 amino acids and eight N-linked glycosylation sites are well tolerated in the C-terminal portion of this domain. Related studies on the hypervariable domain of the amphotropic MuLV SU in an otherwise ecotropic *env* have recently been reported (53). In that study, progressive deletions from the C terminus of the hypervariable domain had

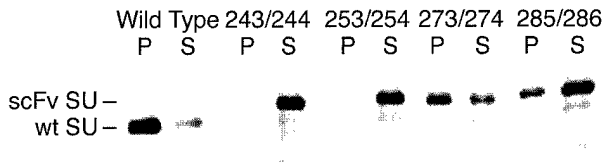

FIG. 5. Particle association of 5145A scFv insert SUs. 3T3 cells producing the indicated viruses were labeled with [^{35}S]cysteine, and culture supernatants were separated into soluble (S) and particulate (P) fractions by sedimentation. Samples were analyzed by RIP with hyperimmune anti-gp70 serum, followed by SDS-PAGE and autoradiography.

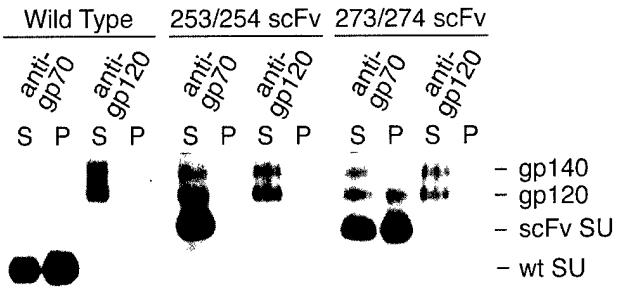

FIG. 6. gp120 binding by 5145a scFv insert SUs. 3T3 cells producing the indicated viruses and SEC-CHO cells secreting HIV-1 gp120 and gp140 were labeled with [^{35}S]cysteine. Culture supernatants were mixed as indicated, and virus particles were separated from soluble proteins by sedimentation. Samples were analyzed by RIP with hyperimmune anti-gp70 serum or human anti-HIV-1 serum, followed by SDS-PAGE and autoradiography.

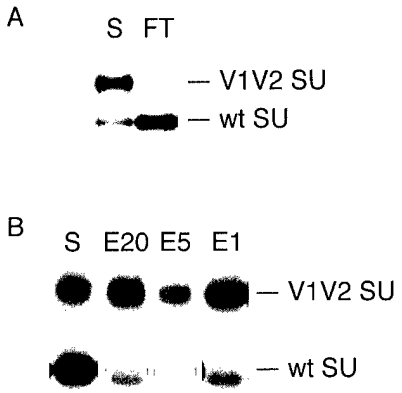

FIG. 7. Separation of retroviral particles with a MAb specific for an insert in SU. Mixtures of wild-type and V1/V2$_{CaseA2}$ 273/274 chimeric viruses were subjected to negative enrichment with MAb SC258 at 5 μg/ml on Pansorbin (A) or positive enrichment with SC258 at the indicated concentrations and His$_6$-protein (A) on Ni^{2+}-NTA resin (B). Virus mixtures before and after enrichment were expanded in 3T3 cells, and [^{35}S]cysteine-labeled culture supernatants were analyzed by RIP with hyperimmune anti-gp70 serum, followed by SDS-PAGE and autoradiography. S, starting mixture; FT, virus not removed by Pansorbin; E20, virus eluted from Ni^{2+}-NTA when SC258 was used at 20 μg/ml; E5, virus eluted from Ni^{2+}-NTA when SC258 was used at 5 μg/ml; E1, virus eluted from Ni^{2+}-NTA when SC258 was used at 1 μg/ml.

little effect on viral growth until over 60% of the domain was removed, and tolerance for small insertions was demonstrated.

In the Fr-MuLV SU studied here, the hypervariable domain consists of 41 amino acids, residues 244 to 284. The N-terminal section of this domain appeared to be more sensitive to insertion than the C-terminal region. Seven-amino-acid insertions (ASAVAGA) were well tolerated at the beginning of the domain (between residues 243 and 244), but large insertions were not (Fig. 2 and 4). Even small insertions had a significant deleterious effect when they were placed 10 residues from this end (between residues 253 and 254) (Fig. 3). In contrast to the relative sensitivity of the N-terminal region of the hypervariable domain, even large inserts had no effect when they were placed following residues 263 or 273 and only a minor effect when placed following residue 285 at the C-terminal boundary of the domain (Fig. 5). In all cases, the biochemical defect associated with the insertions was destabilization of the interaction between SU and TM, but the Envs appeared to fold and be processed efficiently (Fig. 3 and 5). This was consistent with the elevated shedding of SU reported for other alterations in the hypervariable domain (53), in the conserved proline-rich domain (8, 53), and at a highly conserved glycan attachment site in the adjacent, N-terminal region of the C-terminal domain (at residue 302 in Fr-MuLV SU) (19). These observations suggest that the hypervariable domain is situated between sites in the end of the N-terminal domain and beginning of the C-terminal domain of SU that are involved in its interaction with TM.

Despite the sensitivity of the 253/254 site within the hypervariable domain to even the small insertion, substitution of residues 244 to 273 with the same seven-residue sequence had little or no impact on Env function. The 7-amino acid sequence could also substitute for residues 264 to 285 without deleterious effect. The ability to delete all regions of the hypervariable domain argues strongly that this domain does not contain any specific sequence or structure that is essential for Env function. This conclusion is consistent with the extensive sequence and length differences seen for this domain in natural isolates. Hypervariable domains containing as few as 30 residues have been reported (42), and the maximum deletion examined here retained 12 residues of the domain and had an additional 7 residues of foreign sequence. A structural requirement for a spacer between the globular domains of SU seems likely, given the loss of viral titer reported for deletions that retained fewer than 18 residues of the amphotropic hypervariable domain (53). These data are most consistent with a view of the linker as a flexible domain that allows the specific interactions among the N- and C-terminal domains of SU and TM needed to assemble and maintain the active structure of the Env complex. Only changes that interfere with these interactions external to the hypervariable domain would impair envelope function.

Not only are large insertions well tolerated within the hypervariable domain, but coherent structural domains that are inserted can fold into native conformations and can be effectively presented on the surface of the retroviral particle. An SU with an scFv insertion, which itself contains no internal disulfide bonds and carries no glycans, was able to bind antigen when on virus particles (Fig. 6); and an SU with an insert of the 96-amino-acid V1/V2 domain of HIV-1 gp120, which contains two disulfide bonds and eight N-linked glycans, allowed removal of virus particles from suspension by using a MAb directed against a conformational epitope in the V1/V2 domain (Fig. 7A).

These properties of insertions in the hypervariable domain of MuLV SU allowed development of a retroviral particle display system. Bacteriophage particle display systems are not suitable for expression of protein domains whose proper folding is dependent on the glycosylation or other activity found only in eukaryotic cells. An analogous system based on expression in mammalian cells would allow enrichment for variants of such domains. Two types of enrichments might be performed with such a particle display system. Isolation of a sequence with a desired binding activity requires a positive enrichment, in which particles that bind to a specific ligand are preferentially recovered. Isolation of variant sequences that have lost the ability to bind to a specific ligand requires a negative enrichment or depletion protocol in which particles that bind are preferentially removed. Methods for both types of enrichment were demonstrated for MuLV particles carrying the V1/V2 insert in SU, using the MAb directed against a conformational epitope on the insert (Fig. 7). Greater than 30-fold positive enrichment or 400-fold negative enrichment was achieved in a single step of selection and amplification, suggesting that as few as four cycles of enrichment would allow isolation of sequences present in a library at 10^{-6}. Cycling the enrichment procedure should not present a problem, since the 273/274 site

insertions are extremely stable, showing no accumulation of deleted genomes after five cycles of passage through 3T3 cells (data not shown). As constituted, the retroviral particle display system might allow directed modification of complex immunogens that present both desirable and undesirable epitopes, enriching against modified sequences that present the undesirable epitopes and for sequences that continue to express the desirable epitopes in alternation. This system could also be used to isolate small glycopeptides that interact specifically with particular ligands.

An ongoing problem in the use of retroviral vectors for human gene therapy is the lack of target cell specificity afforded by the amphotropic MuLV Env used in most systems (43). Much effort has therefore been put into engineering retroviral Envs to express binding activities that can be used to direct infection to cells of choice, the most successful of which used a 16-residue collagen-binding peptide inserted into an avian retroviral Env (48). Previous attempts with large inserts or substitutions used sites in the N terminus of SU. These constructs lost normal Env function, often required wild-type Env for incorporation into virions, and resulted in low transducing efficiencies (6, 9, 13, 21, 44, 47). The tolerance of the hypervariable domain of SU to large insertions that present new binding activities on the particle surface suggests that expression of ligands at this site in SU may lead to more efficient targeted vector delivery. This use of scFvs would provide a powerful method for targeting a wide range of cell types (47).

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PROVISIONAL APPLICATION

UNDER 37 CFR 1.53(b)(2)

TITLE: **HIV-1 gp120 V1/V2 DOMAIN EPITOPES CAPABLE OF GENERATING NEUTRALIZING ANTIBODIES**

APPLICANT: **ABRAHAM PINTER**

"EXPRESS MAIL" Mailing Label Number EM504554675US

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Lisa C Gray

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HIV-1 gp120 V1/V2 Domain Epitopes Capable of Generating Neutralizing Antibodies

Background of the Invention

There is presently a dearth of candidate HIV vaccines that are considered suitable for wide-scale testing in humans, particularly when considering vaccines capable of inducing protective humoral immunity. Whereas live, attenuated viruses may provide protection against more pathogenic strains, safety considerations are likely to preclude the wide-spread use of such vaccines. A difficulty with purified envelope subunit vaccines is that while the best of these have been able to induce neutralizing responses against the vaccine strain and related laboratory-adapted, T cell-tropic HIV-1 strains, these vaccines have not induced neutralizing responses to primary viruses and clinical HIV-1 isolates (Hanson, 1994; Mascola et al., 1994; Matthews, 1994). This finding may be related to the general resistance of primary viruses to neutralization by sCD4 (Ashkenazi et al., 1991; Gomatos et al., 1990), monoclonal antibodies (D'Souza et al., 1995; Moore et al., 1995), and immune sera from many HIV-infected patients (Golding et al., 1994). The reason for the difference in sensitivities of primary viruses and lab isolates is not clear. It has been suggested that epigenetic factors related to the cells used to prepare the virus (Sawyer et al., 1994) and to the incorporation of host cell adhesion proteins into virion membranes (Guo and Hildreth, 1995; Hildreth and Orentas, 1989) may be involved.

Whereas it is known that some people possess potent neutralizing antibodies against primary strains of HIV, such activities are rare, and the nature of the epitopes that mediate this activity are generally unknown. A major difference between the immune responses of naturally infected individuals and people vaccinated with envelope subunit proteins is that while the humoral responses of the former are directed mostly against conformational epitopes in the viral envelope proteins that are well exposed on native virions (Moore and Ho, 1993), the vaccinees produced mostly antibodies against linear epitopes that were poorly accessible on both monomeric and cell-associated gp120

molecules (VanCott et al., 1995). The natural immune response against HIV-1 has been characterized by isolation and characterization of monoclonal antibodies (mabs) from infected people. These studies have utilized cell-adapted laboratory strains of HIV-1, and the mabs that have been described all have preferential neutralizing activity for lab strains over primary viruses. The major neutralization targets recognized in these studies were the V3 loop and the CD4-binding site (Chamat et al., 1992; D'Souza et al., 1994; Gorny et al., 1993; Thali et al., 1992; Tilley and Pinter, 1993).

Whereas it has been reported that some anti-V3 mabs can neutralize primary viruses (Conley et al., 1994), such neutralization is relatively inefficient, requiring 10-100 ug/ml of antibody (D'Souza et al., 1995), considerably more than that required for neutralization of susceptible lab strains. Consistent with these findings are results showing that depletion of anti-V3 antibodies from a human serum resulted in loss of neutralizing activity against the T-tropic MN strain, but not against several primary isolates (VanCott et al., 1995). This may be related to othe evidence showing that the V3 loop in primary viruses may be buried, and not readily accessible to neutralizing antibodies (Bou-Habib et al., 1994).

A number of human mabs described in the above studies compete for binding of CD4 and have potent neutralizing activities for lab strains of HIV (Cordell et al., 1991; Ho et al., 1991; Tilley et al., 1991). These mabs are directed against conserved, conformational epitopes that are composed of residues scattered over many conserved regions of gp120 (Thali et al., 1992), including residues essential for binding of CD4 itself (Olshevsky et al., 1990). Primary viruses are much less sensitive to neutralization by these mabs than lab strains (Honnen et al., 1996; Moore et al., 1995), similar to their resistance to sCD4 itself, and there have been reports that in some cases these antibodies actually enhance infection by primary HIV-1 isolates (Lee et al., 1997; Schutten et al., 1995; Stamatatos et al., 1997).

Several human mabs against other Env epitopes have been identified that have better neutralizing activities for primary isolates (Trkola et al., 1995). These include IgG

b12, an anti-CD4-binding site human mab isolated from a combinatorial phage library (Burton et al., 1994), 2F5, directed against a linear epitope in gp41 (Conley et al., 1994; D'Souza et al., 1995; Muster et al., 1994; Trkola et al., 1995) and 2G12, directed against a poorly defined, glycan-dependent epitope in gp120 (Fouts et al., 1997; Trkola et al., 1996). The ability of all three of these mabs to neutralize primary viruses is a reflection of their overall increased potencies, but they in fact also have preferential activity for lab strains over primary viruses (Honnen et al., 1996).

Several studies document the role of the V1/V2 domain as a major antigenic target for HIV-1. A number of rodent mabs have been isolated from animals immunized with recombinant IIIB gp120 that are directed against linear (Fung et al., 1992) and conformational epitopes in the V2 domain (Ho et al., 1991; McKeating et al., 1993; Moore et al., 1993). HIV-infected humans have been shown to produce antibodies against linear epitopes located in both the V2 (Kayman et al., 1994; McKeating et al., 1993; Moore et al., 1993) and V1 regions (Honnen et al., 1996; Pincus et al., 1994). The linear V1 epitopes and some of the linear V2 epitopes mediate type-specific neutralization of IIIB virus and related lab strains.

Many of the anti-V2 neutralizing antibodies that have been described were directed against type-specific epitopes and appear to possess weak neutralizing activities. Thus, the significance of these antibodies for *in vivo* protection is unclear. Recently, however, several primate mabs have been described which have more interesting neutralizing properties.

Evidence for the role of the V1/V2 domain in neutralization of HIV-1 comes from recent our own studies with chimpanzee mab C108G, an antibody directed against a glycan-dependent epitope in V2 (Honnen et al., 1996; Vijh-Warrier et al., 1996; Warrier et al., 1994; Wu et al., 1995). This antibody possesses extremely potent neutralizing activities for both lab strains and primary isolates bearing the C108G epitope, including NL-HX-ADA, a primary-like macrophage-tropic isolate.

Summary of the Invention

The invention features a protein which includes a gp120 V1/V2 domain of an HIV-1 strain and not a gp120 V3 domain of an HIV-1 strain, which protein does not substantially bind CD4. The gp120 V1/V2 domain of the protein displays an epitope which is recognized by an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

In various embodiments the two different clades are selected from the group consisting of clade A, clade B, clade C, clade D, and clade E; the V1/V2 domain epitope is recognized by an antibody which neutralizes at least two HIV-1 primary isolates of the same clade with a ND_{90} of less than 100 μg/ml; the V1/V2 domain epitope is recognized by an antibody which neutralizes at least at least one HIV-1 primary isolate from each of at least two different clades with a ND_{90} of less than 100 μg/ml; the V1/V2 domain epitope is recognized by an antibody which neutralizes at least one HIV-1 primary isolate of at least three different clades selected from the group consisting of clade A, clade B, clade C, clade D, and clade E, with a ND_{90} of less than 100 μg/ml; the ND_{90} is less than 50 μg/ml; the ND_{90} is less than 20 μg/ml; the ND_{90} is less than 10 μg/ml; the ND_{90} is less than 5 μg/ml; the ND_{90} is less than 1 μg/ml; the V1/V2 domain includes a region that is at least 50%, 75%, or 90% identical to GEIKNCSFNITTSIRDKVQKEYALFY KLDIVPID; the V1/V2 domain is at least 50%, 75%, or 90% identical to VKLTPLCVTLNCIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCS FNITTSIRDKVQKEYALFYKLDIVPIDNPKNSTNYRLISCNTSVITQA (SEQ ID NO: 1); and the protein is a glycoprotein.

The invention also features a protein which includes a gp120 V1/V2 domain related region that is at least 50% identical to VKLTPLCVTLNCIDLRNATNATSNS NTTNTTSSSGGLMMEQGEIKNCSFNITTSIRDKVQKEYALFYKLDIVPIDNPKNS TNYRLISCNTSVITQA (SEQ ID NO: 1) and not not a gp120 V3 domain of an HIV-1 strain, which protein does not substantially bind CD4. The gp120 V1/V2 domain related

region displaying an epitope which is recognized by an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

The invention also features a protein which includes a gp120 V1/V2 domain of an HIV-1strain and not a gp120 V3 domain of an HIV-1 strain, which protein does not substantially bind CD4. The protein, when used to immunize a rat, being capable of eliciting an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml. In various preferred embodiments the antibody elicited neutralizes at least two HIV-1 primary isolates, at least two HIV-1 primary isolates of two different clades (e.g., clade A, clade B, clade C, clade D, and clade E).

The invention also features a monoclonal antibody which binds the gp120 V1/V2 domain of HIV-1 strain Case-A2 and neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml. In various preferred embodiments the antibody neutralizes at least two HIV-1 primary isolates, at least two HIV-1 primary isolates of two different clades (e.g., clade A, clade B, clade C, clade D, and clade E)

The invention also features a method for stimulating the formation of antibodies capable of neutralizing infection by an HIV viral isolate in at least one mammalian species, which comprises immunizing a mammalian subject with a composition comprising a protein which includes a gp120 V1/V2 domain of an HIV-1 strain and not a gp120 V3 domain of an HIV-1 strain, which protein does not substantially bind CD4. The gp120 V1/V2 domain of the protein displays an epitope which is recognized by an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml. In various preferred embodiments the composition is suspended in a pharameutical carrier or vehicle; the composition comprises an adjuvant; the adjuvant is an aluminum salt; the adjuvant is an oil-in-water emulsion comprising a emulsifying agent and a metabolizable oil; and the composition is administered to the mammalian subject by injection.

The invention also includes a hybrid protein having a first part and a second part, the first part including the protein of claim 1, the second part including an amino terminal

carrier protein comprising all or a portion of Friend MuLV gp70, preferably amino acids 1-33 or 1-263 of gp70.

As used herein, the term "transfected cell" means any cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid encoding a polypeptide of the invention.

As used herein, both "protein" and "polypeptide" mean any chain of amino acid residues, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). The polypeptides of the invention are referred to as "substantially pure," meaning that they are at least 60% by weight (dry weight) the polypeptide of interest. Preferably, the polypeptide is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, the polypeptide of interest. Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The polypeptide can be a naturally occurring, synthetic, or a recombinant molecule consisting of a hybrid with one portion, for example, being encoded by all or part of a Tango-63 gene, and a second portion being encoded by all or part of a second gene.

In the context of a polypeptide or protein, the term "substantially identical" refers to a polypeptide having a sequence that is at least 85%, preferably at least 90%, more preferably at least 95%, and most preferably at least 98% or 99% or more identical to the amino acid sequence of the reference polypeptide. For polypeptides, the length of the reference polypeptide sequence will generally be at least 16 amino acids, at least 20 amino acids, at least 25 amino acids, or preferably at least 35 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, at least 60 nucleotides, at least 75 nucleotides, or at least 90 nucleotides.

Sequence identity can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705) with the default parameters specified therein.

In the case of polypeptide sequences that are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Where a particular polypeptide is said to have a specific percent identity to a reference polypeptide of a defined length, the percent identity is relative to the reference polypeptide. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

The members of a pair of molecules (for example, an antibody-epitope pair or a receptor-ligand pair) are said to "specifically bind" to each other if they bind to each other with greater affinity than to other molecules. Thus, an antibody which specifically binds to a particular epitope within a V1/V2 domain binds to that particular V1/V2 domain epitope with greater affinity than to other V1/V2 domain epitopes.

The amino acid sequences of many HIV-1 gp120 protein are described in Meyers et al., 1996).

The V1/V2 domain is that region of HIV-1 gp120 which corresponds to the following sequence from Case-A2 gp120:

VKLTPLCVTLNCIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCSFNITTSIR DKVQKEYALFYKLDIVPI DNPKNSTNYRLISCNTSVITQA (SEQ ID NO: 1)

Other V1/V2 domains can be identified by aligning SEQ ID NO: 1 with a gp120 sequence using standard sequence alignment software. Myers et al. (1996) provides alignments of a number of gp120 proteins. The four Cys residues underlined in SEQ ID NO:1 are essentially invariant and can be used to assist in alignment. Other important

highly conserved residues are the underlined Ser, Phe, Ala, and Asp residues. It should be noted that the V1/V2 domain defined above extends somewhat beyond the V1 and V2 loops as defined in Myers et al. (1996).

The "V3 domain" of gp120 is that region identified in Myers et al. (1996) as the V3 loop.

A protein which does not substantially bind to CD4 is a protein which does not show appreciable binding of CD4 when tested in a CD4 binding assay such as that described in U.S. Patent 5,653,985.

The antigenic peptides described herein are useful as vaccine compositions. They may also be used in immunoassays for anti-HIV antibodies and for the production of anti-HIV antiserum.

The invention encompasses nucleic acid molecules encoding the proteins of the invention. Nucleic acid molecules within the invention can be cDNA, genomic DNA, synthetic DNA, or RNA, and can be double-stranded or single-stranded (*i.e.*, either a sense or an antisense strand). Fragments of these molecules, which are also considered within the scope of the invention, can be produced, for example, by the polymerase chain reaction (PCR) or generated by treatment with one or more restriction endonucleases. A ribonucleic acid (RNA) molecule can be produced by *in vitro* transcription.

The preferred methods and materials are described below in examples which are meant to illustrate, not limit, the invention. Skilled artisans will recognize methods and materials that are similar or equivalent to those described herein, and that can be used in the practice or testing of the present invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference

in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Detailed Description of the Invention

Drawings

Figure 1: Comparison of the sequence of HIV-1 Case A2 gp120 and HIV-1 HXB2 between residues 158 and 185 to the most common and second most common residues found at these positions in 55 clade B strains. The frequency of occurrence of a particular residues is shown below the consensus sequences.

Figure 2: Proposed structure of the Case A2 fusion protein. Amino acids 111-206 of the HIV-1 Case A2 gp120 V1/V2 domain were fused to the C-terminus of a 263 amino acid N-terminal fragment of MuLV gp70 protein. The Q residue at position 9 of MuLV gp70 was replaced by five His residues to provide an affinity tag for ready purification of the fusion protein.

Figure 3: Schematic illustration of the serum fractionation protocol. The three affinity columns used in the serum fractionation protocol are shown at the left. Just to the right of each column is a graph illustrating the amount of antibody recovered by each wash of the corresponding column.

Figure 4A: Neutralization of Bal (squares; ND_{50} = 0.24 μg/ml), NHA (diamonds; ND_{50} = 0.096 μg/ml), ADA (circles; ND_{50} = 0.058 μg/ml), Th014B (upward pointing triangles; ND_{50} = 0.14 μg/ml), Ug/005D (downward pointing triangles; ND_{50} = 0.03 μg/ml), and Th024E (crosses; ND_{50} = n.d.) by antibodies eluted from the Case-A2 fusion protein affinity column with the 5M GuHCl wash.

Figure 4B: Neutralization of Bal (squares; ND_{50} = 12.0 μg/ml), NHA (diamonds; ND_{50} = 9.9 μg/ml), ADA (circles; ND_{50} = 3.2 μg/ml), Th014B (upward pointing triangles;

ND_{50} = 0.86 μg/ml), Ug/005D (downward pointing triangles; ND_{50} = <0.8 μg/ml), and Th024E (crosses; ND_{50} = 3.8 μg/ml) by antibodies eluted from the T15K peptide affinity column with the pH 3 wash.

Figures 5A-5D: Cross-clade immmunoreactivity of sera of maques immunized with Case-A2 fusion protein (p565; Figures 5B, 5C, and 5D) or gp70 carrier protein (p621; Figure 5A). Sera were obtained two weeks after a second immunization and tested by direct ELISA against gp70 carrier protein (open squares), Case-A2 fusion protein (closed squares), Thai clade E (p580, Tho6.05E) V1/V2 fusion protein (circles), Brazilian clade B (p599, 92Br14.01B) V1/V2 fusion protein (triangles), and T15K peptide (cross-hatched squares).

Proteins Which Elicit Highly Neutralizing Antibodies

The proteins of the present invention display conformational epitopes which elicit highly neutralizing antibodies which can neutralize primary HIV-1 isolates. Preferred proteins are those which can elicit antibodies which neutralize primary isolates in two or more different clades (e.g., two or more of clades A, B, C, D, and E).

The proteins of the present invention can exist in a variety of different conformations. Thus, when a protein composition is said to comprise a protein which express a particular epitope, it does not mean that every protein molecule in the composition displays that particular epitope. A protein of a given sequence can exist in a variety of conformations, and a variety of conformations are likely to be present in any composition containing a given protein. The proteins of the invention are expressed and isolated such that at least 10%, preferably 20%, 50%, 70%, or even 90% of the protein molecules display the desired epitope.

Case-A2 gp120 V1/V2 Domain Fusion Protein Elicits Highly Neutralizing Antibodies

The studies described below demonstrate that a fusion protein containing the V1/V2 domain of gp120 derived from the Case-A2 clinical HIV-1 isolate can generate highly neutralizing antibodies when used to immunize rats. Significantly, the fusion protein can generate antibodies that neutralize a number of primary macrophage-tropic

HIV strains. Moreover, the fusion protein can generate antibodies which neutralize primary HIV isolates of several different clades.

The fusion protein containing the V1/V2 domain of gp120 used in the studies described below (the "Case-A2 fusion protein") consists of residues 111-206 of the gp120 protein of the Case A2 isolate of HIV-1 (Wang et al., 1995) joined to the C-terminus of residues 1-263 of MuLV gp70 protein (Kayman et al., 1994). For ease of purification, a His6 affinity tag was inserted into residues 1-263 of MuLV gp70 by replacing the Q residues at position 9 with five His residues. The Case-A2 V1/V2 domain has the following sequence:

VKLTPLCVTLNCIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCSFNITTSIR DKVQKEYALFYKLDIVPI DNPKNSTNYRLISCNTSVITQA (SEQ ID NO: 1)

To create a Case-A2 fusion protein expression vector, a recombinant gene encoding the Case-A2 fusion protein was inserted into the pEE14 expression vector (Celltech Limited, Berkshire, UK). This construct contains two extraneous amino acids (AS) between the gp70 and V1/V2 sequences and two extraneous amino acids (GA) after the C-terminus of the V2 region. This expression vector also expresses the glutamine synthetase gene, which allows selection of transfected cells by growth in glutamine-deficient medium in the presence of methionine sulfoxamine (MSX), a glutamine antagonist (Bebbington et al., 1992). The Case-A2 fusion protein expression vector was transfected into CHO cells, and transfected clones were isolated in the selecting medium (glutamine-free RPMI containing 10% dialyzed fetal bovine serum and 80 μM MSX). Clones expressing the Case-A2 fusion protein were identified by ELISA using mab 238, directed against a conformational epitope in V2 (Moore et al., 1993). Proteins secreted by several postive clones were analyzed by radioimmunoprecipitation and SDS-PAGE.

Analysis of the Case-A2 fusion protein produced as described above revealed that the protein exists in at least two forms which can be distinguished by their reactivity with different monoclonal antibodies. Two monoclonal antibodies, C9B6 and K19B3, isolated in our lab from rats immunized with the Case-A2 fusion protein and directed against

linear HIV-1 gp120 epitopes recognized approximately half of the fusion protein molecules, while two mouse monoclonal antibodies, 684-238 and SC258 (obtained from Abbott Labs) directed against conformational V2 epitopes (Wu et al., 1995), reacted predominantly with the other half of the fusion protein molecules. This suggests that about half of the molecules were correctly folded and presented native conformational epitopes, while the other half of the molecules may be misfolded and present linear or misfolded epitopes.

Immunizations of Rats with Case-A2 Fusion Protein

A number of 2-4 month old female Fischer F344 rats were immunized with purified Case A2 fusion protein or HXB2 fusion protein. HXB2 fusion protein was created in the same manner as the Case A2 fusion protein except that the V1/V2 domain portion of the fusion protein has the sequence of the HXB2 HIV-1 isolate rather than the Case A2 isolate (Figure 1).

The fusions proteins were combined with either QS21 (from CBCX, Inc. Worcester, MA) or RAS MPL+TDM (Ribi Immunochemicals, Inc. Hamilton, MO) adjuvant according to the manufacturer's instructions. Three rats in each group were immunized with immunogens at 5 ug/rat and were boosted using the same formulation 6 weeks later at 1 μg/rat. Rats were then boosted at a 5-6 week intervals using the same formulation at 1 μg/rat. Rats were bled one week after each boost. Serum was analyzed by ELISA using various antigens.

The ELISA analysis revealed that the Case A2 fusion protein is a more effective immunogen than the HXB2 fusion protein. Sera from both RAS and QS21 adjuvant groups were analysed for their cross-reactivity against purified envelope proteins derived from LAV, MN, and CM strains, using equal amounts of each envelope protein. In general, rats immunized with Case-A2 fusion protein produced higher titers and better cross-reactivity than those immunized with HXB2 fusion protein. All three rats immunized with Case-A2 fusion protein produced antibodies that cross-reacted with LAV gp120, and several of the immunized animal sera demonstrated appreciable titers to MN

and BaL gp120s. These results indicated that despite the purported hypervariability of the V1/V2 region, the Case-A2 fusion protein is able to generate antibodies with significant titers against several unrelated gp120 with heterologous V1/V2 sequences. Furthermore, since the gp120s used were produced in CHO cells, they are likely to be properly folded. Thus, it appears that cross-reactive anti-V1/V2 antibodies produced by rats immunized with Case A2 fusion protein recognize conserved, native epitopes within the V1/V2 domain of gp120.

Epitope Specificity of anti-V1/V2 Antibodies Elicited by Case-A2 Fusion Protein

Western blots assays indicated that the sera of some rats immunized with the V1/V2 fusion proteins contained antibodies that reacted with epitopes in the V1/V2 fusion proteins that are not dependent on the maintenance of disulfide bonds. In order to map the epitopes in the Case-A2 sequence recognized by these antibodies, a set of overlapping 15-mer peptides that represent the entire Case-A2-V1/V2 sequence were prepared. ELISA assays with these peptides showed that, for five of seven rats immunized twice with the immunogen, the linear epitopes recognized were localized to a single peptide, p7, that corresponds to the most highly conserved region of the V2 domain (TABLE 1). Two other rats immunized seven times with Case A2 fusion protein also recognized only peptide p7. In contrast, a screen of of 100 sera of HIV-infected humans identified only one that reacted with peptide p7. This result suggests that this sequence, while highly conserved, is not very immunogenic when expressed during HIV infection, but is immunogenic when presented in the context of the Case A2 fusion protein.

TABLE 1: Reactivity of Case A2 Sera to Various V1/V2 Domain Peptides

Peptide	Sequence	OD_{405} (range)
p1	asVKLTPLCVTLNSI (SEQ ID NO:2)	0.05 (0-0.38)
p2	VTLNCIDLRNATNAT (SEQ ID NO:3)	0.01 (0-0.09)
p3	ATNATSNSNTTNTTS (SEQ ID NO:4)	0.01 (0-0.06)
p4	TNTTSSSGGLMMEQG (SEQ ID NO:5)	0.02 (0-0.16)
p5	MMEQGEIKNCSFNIT (SEQ ID NO:6)	0.00
p6	SFNITTSIRDKVQKE (SEQ ID NO:7)	0.00
p7	SIRDKVQKEYALFYK (SEQ ID NO:8)	1.04 (0.39-1.46)
p8	EYALFYKLDIVPIDN (SEQ ID NO:9)	0.00

In order to further characterize the antibodies elicited by Case A2 fusion protein, analogues of peptide p7 were generated. To increase the solubility of the analogues and to facilitate the immobilization of the analogues, two additional lysine residues were introduced at the N-terminus, followed by two naturally occurring threonines. The resulting peptide was called T15K, and has the sequence kkTTSIRDKVQKEYALFYK (SEQ ID NO:10).

The specificities of the antibodies recognizing this peptide's epitopes were further defined by analyzing a series of N-terminal and C-terminal truncations of peptide T15K peptide (TABLE 2). Peptides that expressed the epitope, as defined by retention of reactivity by the majority of rat sera, were T15K, pep1, pep2, and pep4. Deletion of the C-terminal lysine did not affect the epitope, while deletion of an additional two hydrophobic residues, phenylalanine and tyrosine, resulted in a reduction of binding. Deletion of an additional leucine resulted in loss of recognition by all but one serum. N-terminal deletions of the two threonines retained reactivity, while further deletion of serine and isoleucine led to the complete loss of reactivity of both sera. Thus, the minimal epitopes recognized were located in the sequence (S)IRDKVQKEYAL(FY)

(SEQ ID NO:11), with a decreased or unknown effect of the terminal residues in parentheses. Interestingly, this sequence partially overlapped with the homologous peptide determinant (STSIRGKV; SEQ ID NO:12) of the strongly neutralizing C108G MAb (Wu et al., 1995), suggesting that this region may also be a neutralization epitope.

TABLE 2: Epitope Mapping of Antiboides Elicited by Case A2 Fusion Protein

Peptide	Sequence		OD_{405} (range)*
C-terminal deletions			
T15K	kkTTSIRDKVQKEYALFYK	(SEQ ID NO:13)	1.34 (0.91-1.92)
pep1	kkTTSIRDKVQKEYALFY	(SEQ ID NO:14)	0.86 (0.49-1.53)
pep2	kkTTSIRDKVQKEYAL	(SEQ ID NO:15)	0.59 (0.02-1.40)
pep2a	kkTTSIRDKVQKEYA	(SEQ ID NO:16)	0.35 (0.02-1.38)
pep3	kkTTSIRDKVQKEY	(SEQ ID NO:17)	0.23 (0.02-0.81)
N-terminal deletions			
pep4	kk--SIRDKVQKEYALFYK	(SEQ ID NO:18)	0.99 (0.02-1.40)
pep5	kk----- RDKVQKEYALFYK	(SEQ ID NO:19)	0.24 (0.02-0.94)
pep6	kk-------DKVQKEYALFYK	(SEQ ID NO:20)	0.09 (0.02-0.38)

*Values are average of six rats. ELISAs were performed with 1:100 serum dilutions.

Neutralization Activities of Anti-V1/V2 Antibodies Elicited by Case A2 Fusion Protein

Preliminary experiments indicated that the sera of all of the immunized rats possessed neutralization activities against a number of HIV-1 isolates. In order to evaluate the role and potency of the anti-V1/V2 antibodies present in these sera, and to eliminate background effects due to nonspecific components present in the sera, the immunoglobulins were sequentially fractionated by affinity chromatography using several different antigen columns (Figure 3). The serum was first absorbed on a column containing p621, a recombinant protein containing only the gp70-derived sequences (including the His6 tag) present in the fusion protein, to remove the irrelevant anti-gp70

antibodies. The material which did not adhere to this column was applied to a column containing immobilized T15K peptide, and the unabsorbed flow-through of the T15K column was then applied to a column containing immobilized Case A2 fusion protein. . Material which bound to the T15K peptide column or to the Case-A2 V1/V2 column were eluted sequentially with low pH buffers and then with the denaturant 5M guanidium hydrochloride (GuHCl), as described below. This permitted the separate isolation of T15K peptide-specific antibodies and antibodies specific for conformational V1/V2 epitopes.

Immunoaffinity columns containing the T15K peptide, the p621 gp70 fragment, or complete Case-A2 V1/V2 fusion protein were prepared by incubating cyanogen bromide-activated Sepharose beads (Pharmacia) at 4°C with PBS solutions containing 2 mg of protein or 4.5 mg of peptide per ml of beads for 24 hrs. Excess binding sites were blocked by treatment with 100mM pH8.0 Tris buffer, the beads were washed, and used for antibody fractionations. Sera were diluted five to ten-fold with PBS and shaken overnight with the beads at a ratio of approximately 0.4 ml of serum per ml of beads. The beads were packed into a column, and flow-through containing depleted serum was collected. The column was then washed with PBS and eluted with 10 mls of either pH 2.5 glycine-HCl buffer or sequentially with pH 3.0 and 1.0 glycine-HCl buffers followed by 5M guanidine hydrochloride (GuHCl). The low pH eluates were neutralized immediately with the appropriate volume of 2M tris, pH 9.2 buffer; GuHCl was removed by extensive dialysis against PBS. To stabilize the purified antibodies they were reconstituted to 100% fetal bovine serum; this was done by adding a volume of fetal bovine serum equivalent to the desired final volume and concentrating the sample back to the initial volume of the serum sample, using Centricon-50 spin columns. The eluted antibodies were then analyzed for antigen specificity and for neutralizing activity. In some cases, samples were first run over a control column containing similar amounts of immobilized BSA; this did not result in depletion of any antibody activity or the recovery of specific antibody activity in the eluted fractions.

A total of 1.4 mg of antibody was recovered from the serum of one rat immunized seven times at roughly monthly intervals. Approximately 63% of the antibody was directed against gp70, about 24% was directed against T15K peptide, and about 13% of the the antibody was directed against conformational epitopes (i.e., bound tightly only to the Case-A2 column). Interestingly, a similar fractionation of the serum of another rat immunized only twice gave a higher percentage and yield of antibodies against the conserved conformational epitopes, suggesting that the efficiency of these immunizations would be improved by more appropriate timing of boosts.

The neutralizing activities of these samples were compared to those of the starting serum and protein G-purified IgG sample as follows. HIV-1 neutralizations were measured by a fluorescent focus assay, performed in 3 day PHA-activated cultures of human PBMCs grown in complete RPMI-1640 medium supplemented with 10% fetal bovine serum and 50 units recombinant human IL-2/ml (Boehringer Mannheim Inc.). Virus-containing supernatants were preincubated with different dilutions of antibody for 1 hr at 37°C, after which the virus-antibody mixture was added to 5 x 10^5 PBMCs. Neutralizations were measured at times when 2-5% of the cells in the control culture were infected, as determined by assay of the extent of viral spread in control wells; this generally represented an infection period 4-7 days. To assay for infection cells were plated out on polylysine-coated multi-spot slides at a concentration of 1 x 10^5 cells per 5 mm well, the slides fixed with acetone, and the cells stained by incubation with a biotinylated polyclonal IgG purified from human HIV-positive sera by protein A chromatography, followed by FITC-conjugated strepavidin. Infected cells were quantitated by counting fluorescent cells using a Nikon Diophot microscope equipped for epifluorescence. The number of positive cells were determined in five separate areas containing confluent layers of cells (approximately 1,100 cells per area) and all samples were assayed in duplicate wells, so that approximately 11,000 total cells were examined for each point. Reproducibility in viral end points (ND_{50}s) was high within single

experiments, but variations as much as two-fold in either direction were seen between experiments, particularly when different batches of virus and cells were used.

The antibodies which recognized V1/V2 domain epitopes all possesed potent neutralization activities for a number of primary macrophage-tropic viruses (Figure 4A and Figure 4B). The GuHCl eluate of the Case A2 fusion protein column had the most potent activity (Figure 4A), but the pH3 eluate of the T15K also had significant neutralizing activity (Figure 4B). Both antibody fractions neutralized all primary viruses assayed, including primary isolates of clades B, C, D and E. In each case the anti-Case A2 fusion protein antibody GuHCl eluate fraction was approximately 10-fold more potent that the anti-T15K peptide antibody fraction. Thus, ND50 values were in the range of 0.03-0.24 ug/ml for the anti-Case A2 fusion protein antibody GuHCl eluate fraction (with the exception of Th024E, for which an ND50 was not obtained) and in the range of <0.8-12 ug/ml for the anti-T15K peptide antibody fraction. As an example, these antibody fractions neutralized a primary clade D isolate from Uganda, Ug005-D with ND90 values of 1.4 ug/ml for the anti-T15K antibody fraction and 0.20 ug/ml for the anti-Case A2 fusion protein antibody GuHCl eluate fraction. In addition to the viruses shown in Figure 4A, the GuHCl eluate of the Case A2 fusion protein column also neutralized a clade B clinical isolate, US716B, with an ND50 of 0.03 ug/ml. The T15K pH3 eluate also was able to neutralize this virus, but with about an order of magnitude lower potency (ND50 of < 0.80 ug/ml).

Immunization of Primates

Rhesus macaques can provide an animal model suitable for testing of HIV vaccines. In order to determine whether a primate will produce useful neutralizing antibodies when immunized with Case-A2 fusion protein, three rhesus macaques were immunized with the Case-A2 fusion protein, and one rhesus macaque was immunized with a control immunogen consisting of the gp70-derived portion of the fusion protein, p621. The antigens were formulated with Ribi RAS adjuvant and administered by subcutaneous injection at an initial dose of 5 ug/kg, followed by a boost after 1 month at

a dose of 1 ug/kg. Bleeds were taken on the day of and one week after the initial immunization and at weekly intervals following the boost. Antibodies reactive with the immunogen were detected after the first boost. The animal immunized with the p621 produced antibodies specific for the p621 protein, while the three animals immunized with the Case A2 fusion protein produced antibodies directed against both gp70 portion and the V1/V2 portion of the Case A2 fusion protein.

These antibodies were further characterized by absorption of the gp70-specific fraction on a gp70 column followed by ELISA against several V1/V2 fusion proteins. As shown in Figures 5A-5D, for the animal immunized with the gp70 fragment (p621) the gp70-specific antibodies were all absorbed on an affinity column containing the immobilized p621 protein. For the three animals immunized with the Case A2 fusion protein, the p621 column flow throughs retained reactivity for the V1/V2 portion of the Case A2 fusion protein, but not for p621. All three of these sera recognized two heterologous proteins in addition to the Case A2 sequence, a Brazilian clade protein and a Thai clade E protein (Gao et al., 1994). The titers were higher for the autologous immunogen than for the non-autologous proteins, and lowest for the more distant clade E protein. However, this cross-reactivity demonstrated that these macaques were producing a fraction of antibodies directed against highly conserved V1/V2 epitopes, in addition to antibodies restricted for the Case-A2 and related clade B proteins. Sera from the animal with the highest titer, #7026, also reacted weakly with the T15K peptide.

Preparation of Monoclonal Antibodies

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be prepared using the V1/V2 fusion proteins described above and standard hybridoma technology (see, for example, Kohler et al., *Nature* 256:495, 1975; Kohler et al., *Eur. J. Immunol.* 6:511, 1976; Kohler et al., *Eur. J. Immunol.* 6:292, 1976; Hammerling et al., "Monoclonal Antibodies and T Cell Hybridomas," Elsevier, NY, 1981; Ausubel et al., *supra*).

In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in Kohler et al., *Nature* 256:495, 1975, and U.S. Patent No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al., *Immunology Today* 4:72, 1983; Cole et al., *Proc. Natl. Acad. Sci. USA* 80:2026, 1983), and the EBV-hybridoma technique (Cole et al., "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated *in vitro* or *in vivo*. The ability to produce high titers of mAbs *in vivo* makes this the presently preferred method of production.

Once produced, polyclonal or monoclonal antibodies are tested for specific gp120 recognition by Western blot or immunoprecipitation analysis by standard methods, e.g., as described in Ausubel et al., *supra*. Among the proteins that bind to V1/V2 fusion proteins are C936, K19B3, SC258, and 684-238.

Nucleic Acid Molecules Encoding a Protein of the Invention

The invention includes nucleic acid molecules encoding the proteins of the invention. The nucleic acid sequences can be naturally occurring sequences (e.g., sequences clone from HIV-1 itself) or sequences that differ from those that occur naturally, but, due to the degeneracy of the genetic code, encode the same polypeptide. In addition, these nucleic acid molecules are not limited to sequences that only encode, and thus, can include coding sequence that encodes a carrier polypeptide, as well as some or all of the non-coding sequences, e.g., regulatory sequences.

The nucleic acid molecules of the invention can be synthesized (for example, by phosphoramidite-based synthesis) or obtained from a natural source (e.g., a virus or a recombinant virus).

The isolated nucleic acid molecules of the invention encompass fragments that are not found as such in the natural state. Thus, the invention encompasses recombinant molecules, such as those in which a nucleic acid sequence is incorporated into a vector

(for example, a plasmid or viral vector) or is joined to a second nucleic acid sequence sich that the joined sequences encode a chimeric protein.

The invention also features a vector that includes a nucleic acid molecule encoding a protein of the invention. In various specific embodiments, the vector is an expression vector, and can include a regulatory element such as the cytomegalovirus hCMV immediate early gene, the early promoter of SV40 adenovirus, the late promoter of SV40 adenovirus, the lac system, the trp system, the TAC system, the TRC system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase, the promoters of acid phosphatase, and the promoters of the yeast α-mating factors. The vector can be a plasmid or a virus, such as a retrovirus.

Particularly preferred are expression vectors which express the protein of the invention as part of a fusion protein with gp70. Suitable fusion protein expression vectors are described in U.S. Patent 5,643,756 (Kayman et al.), hereby incorporated by reference.

In another aspect, the invention features a genetically engineered host cell, particularly a eukaryotic cell, which includes a vector, as described above.

Use

The proteins of the invention can be combined with a suitable adjuvant (e.g., an aluminum salt) to create a vaccine. Vaccine formulations will contain an effective amount of the selected protein antigen (i.e., an amount of protein which, when combined with adjuvant, will cause the subject (e.g., chimpanzees, maques, baboons, or humans) vaccinated to produce sufficient specific immunological response to provide for protection against subsequent exposure to HIV. The vaccine compositions may also be used therapeutically treatment of subjects (e.g., chimpanzees, maques, baboons, or humans) already infected with HIV.

In many cases the vaccine will need to be administered more than once to bring about the desire therapeutic or prophylactic effect. The precise protocol (dosage and

frequency of administration can be established through standard clinical trials. Those skilled in the art will be able to design suitable clinical trials using the results of animal trials (e.g., studies conducted in non-human primates). Dosages may range from 0.1 mg/dose to 1 mg/dose, 10 mg/dose, 100 mg/dose, or 250 mg/dose. The effective amount of a given protein will depend on a number of factors including antigenicity and purity.

The antigen and adjuvant are generally suspended in a small volume (generally 2 ml or less) of a pharmaceutically acceptable carrier.

Adjuvants and vaccination protocols are discussed in U.S. Patent No. 5,614,612, hereby incorporated by reference.

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Claims

1. A protein comprising a gp120 V1/V2 domain of an HIV-1 strain and not comprising the gp120 V3 domain of an HIV-1 strain, wherein said protein does not substantially bind CD4, said gp120 V1/V2 domain of said protein displaying an epitope which is recognized by an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

2. The protein of claim 1, wherein said V1/V2 domain epitope is recognized by an antibody which neutralizes at least at least one HIV-1 primary isolate from each of at least two different clades with a ND_{90} of less than 100 μg/ml.

3. The protein of claim 1, wherein said said two different clades are selected from the group consisting of clade A, clade B, clade C, clade D, and clade E.

4. The protein of claim 1, wherein said V1/V2 domain epitope is recognized by an antibody which neutralizes at least two HIV-1 primary isolates of the same clade with a ND_{90} of less than 100 μg/ml.

5. The protein of claim 3, wherein said V1/V2 domain epitope is recognized by an antibody which neutralizes at least one HIV-1 primary isolate of at least three different clades selected from the group consisting of clade A, clade B, clade C, clade D, and clade E, with a ND_{90} of less than 100 μg/ml.

6. The protein of claim 1 wherein said ND_{90} is less than 50 μg/ml.

7. The protein of claim 1 wherein said ND_{90} is less than 20 μg/ml.

8. The protein of claim 1 wherein said ND_{90} is less than 10 μg/ml.

9. The protein of claim 1 wherein said ND_{90} is less than 5 μg/ml.

10. The protein of claim 1 wherein said ND_{90} is less than 1 μg/ml.

11. The protein of claim 1 wherein said V1/V2 domain comprises a region that is at least 50% identical to GEIKNCSFNITTSIRDKVQKEYALFYKLDIVPID.

12. The protein of claim 1 wherein said V1/V2 domain comprises a region that is at least 75% identical to GEIKNCSFNITTSIRDKVQKEYALFYKLDIVPID.

13. The protein of claim 1 wherein said V1/V2 domain comprises a region that is at least 90% identical to GEIKNCSFNITTSIRDKVQKEYALFYKLDIVPID.

14. The protein of claim 1 wherein said V1/V2 domain is at least 50% identical to
VKLTPLCVTLNCIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCSFNITTSIR DKVQKEYALFYKLDIVPIDNPKNSTNYRLISCNTSVITQA (SEQ ID NO: 1).

15. A protein comprising a gp120 V1/V2 domain related region that is is at least 50% identical to
VKLTPLCVTLNCIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCSFNITTSIR DKVQKEYALFYKLDIVPIDNPKNSTNYRLISCNTSVITQA (SEQ ID NO: 1) and not comprising the gp120 V3 domain of an HIV-1 strain, wherein said protein does not substantially bind CD4, said gp120 V1/V2 domain related region displaying an epitope which is recognized by an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

16. The protein of claim 1 wherein said V1/V2 domain is at least 90% identical to

VKLTPLCVTLNCIDLRNATNATSNSNTTNTTSSSGGLMMEQGEIKNCSFNITTSIR DKVQKEYALFYKLDIVPIDNPKNSTNYRLISCNTSVITQA (SEQ ID NO: 1).

17. The protein of claim 1, wherein said protein is a glycoprotein.

18. A protein comprising a gp120 V1/V2 domain of an HIV-1 strain and not comprising a gp120 V3 domain of an HIV-1 strain, wherein said protein does not substantially bind CD4, said protein, when used to immunize a rat, being capable of eliciting an antibody which neutralizes at least one clade B HIV-1 primary isolate and at least one clade D HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

19. Monoclonal antibody which binds the gp120 V1/V2 domain of HIV-1 strain Case-A2 and neutralizes at least one clade B HIV-1 primary isolate and at least one clade D HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

20. The monoclonal antibody of claim 19 wherein said antibody neutralizes at least one clade A HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

21. A method for stimulating the formation of antibodies capable of neutralizing infection by an HIV viral isolate in at least one mammalian species, which comprises immunizing a mammalian subject with a composition comprising the protein of claim 1.

22. The method of claim 21 wherein said composition is suspended in a pharameutical carrier or vehicle.

23 . The method of claim 21 wherein said composition comprises an adjuvant.

24. The method of claim 23 wherein said adjuvant is an aluminum salt.

25. The method of claim 23 said adjuvant is an oil-in-water emulsion comprising a emulsifying agent and a metabolizable oil.

26. The method of claim 21 wherein said composition is administered to said mammalian subject by injection.

27. An nucleic acid molecule encoding the protein of claim 1.

28. An expression vector comprising the nucleic acid molecule of claim 27.

29. A host cell harboring the vector of claim 28.

30. A hybrid protein comprising a first part and a second part, said first part comprising the protein of claim 1, said second part comprising an amino terminal carrier protein comprising all or a portion of Friend MuLV gp70.

31. The protein of claim 30 wherein said portion of gp70 comprises amino acids 1-33 of gp70.

Abstract

The invention features a protein which includes a gp120 V1/V2 domain of an HIV-1 strain and not a gp120 V3 domain of an HIV-1 strain, which protein does not substantially bind CD4. The gp120 V1/V2 domain of the protein displays an epitope which is recognized by an antibody which neutralizes at least one HIV-1 primary isolate with a ND_{90} of less than 100 μg/ml.

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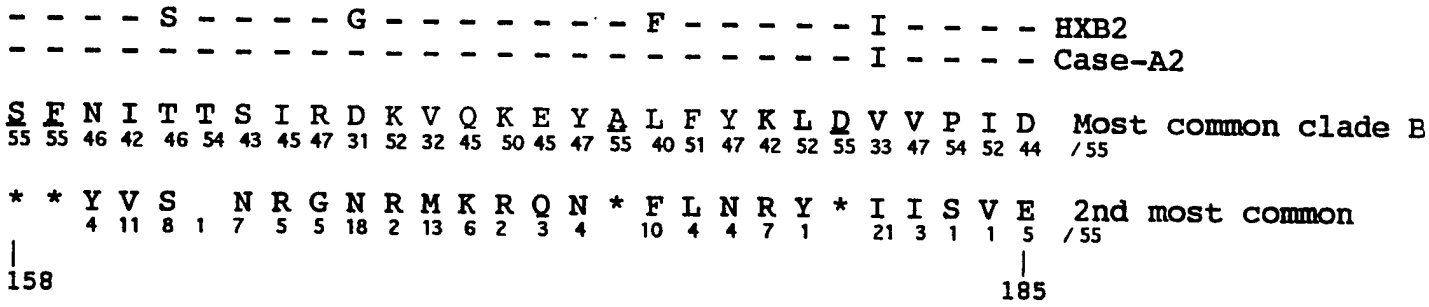
- - - - S - - - - G - - - - - - - - F - - - - - - I - - - - HXB2
- I - - - - Case-A2
S F N I T T S I R D K V Q K E Y A L F Y K L D V V P I D Most common clade B
55 55 46 42 46 54 43 45 47 31 52 32 45 50 45 47 55 40 51 47 42 52 55 33 47 54 52 44 /55
* * Y V S N R G N R M K R Q N * F L N R Y * I I S V E 2nd most common
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158
185

FIGURE 1

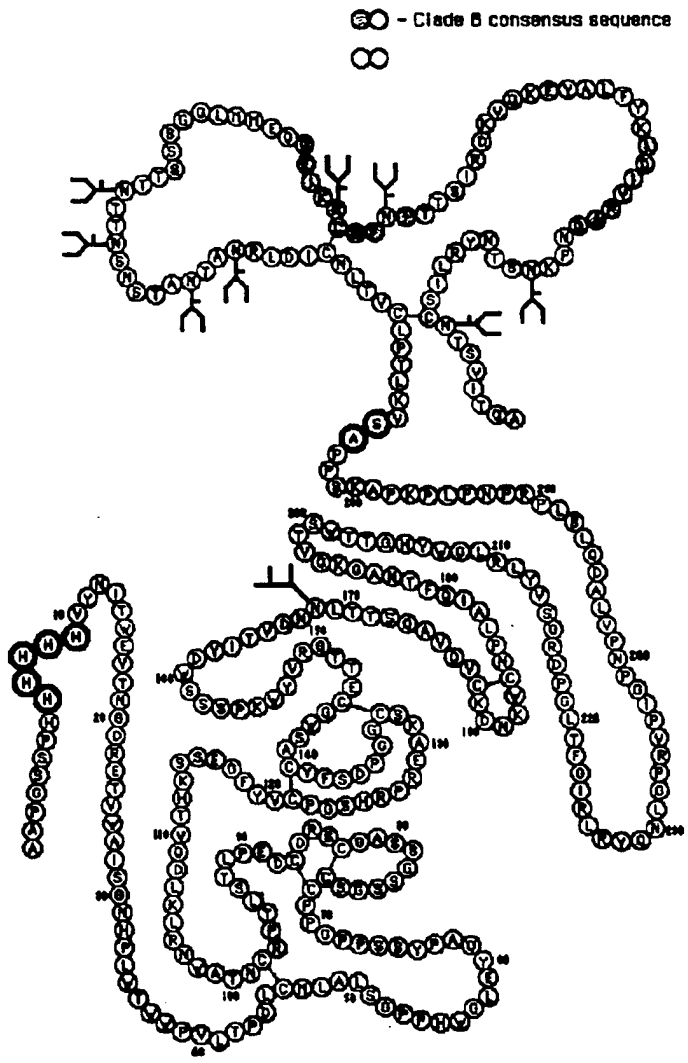


FIGURE 2

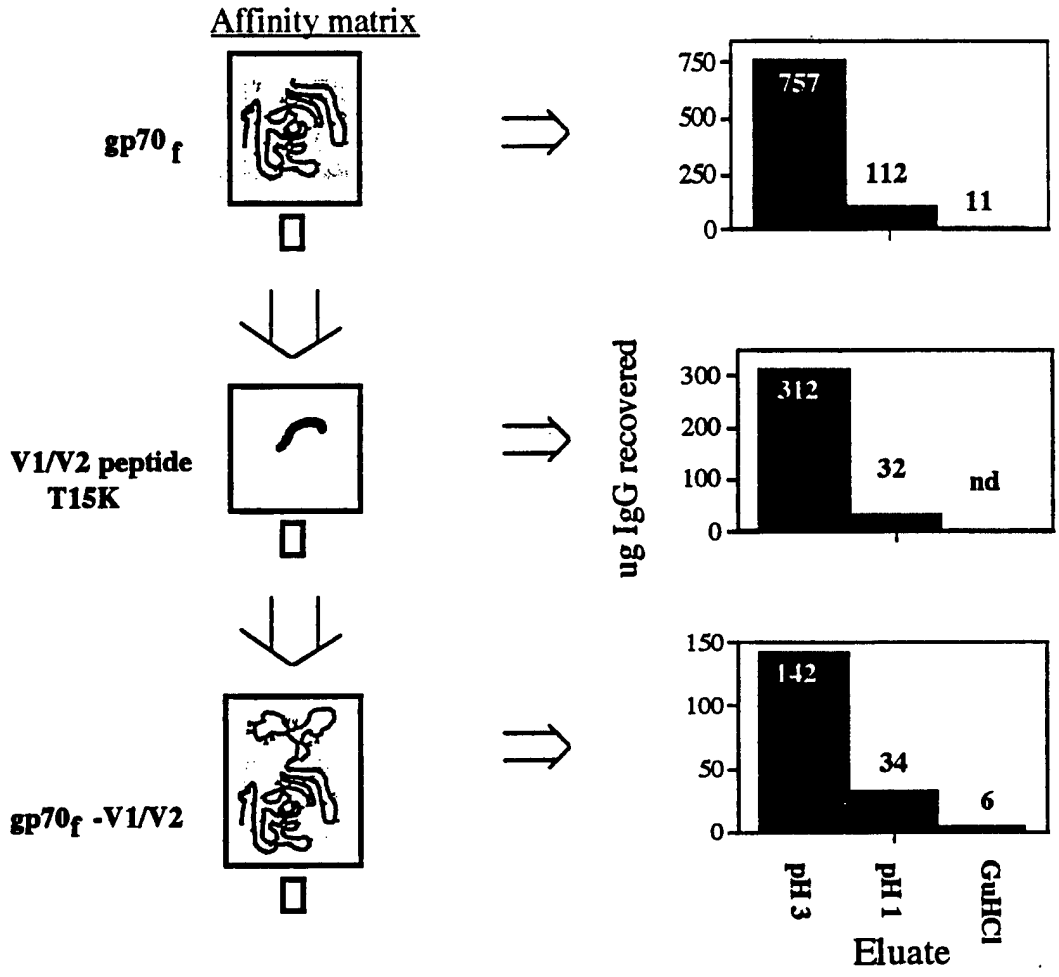
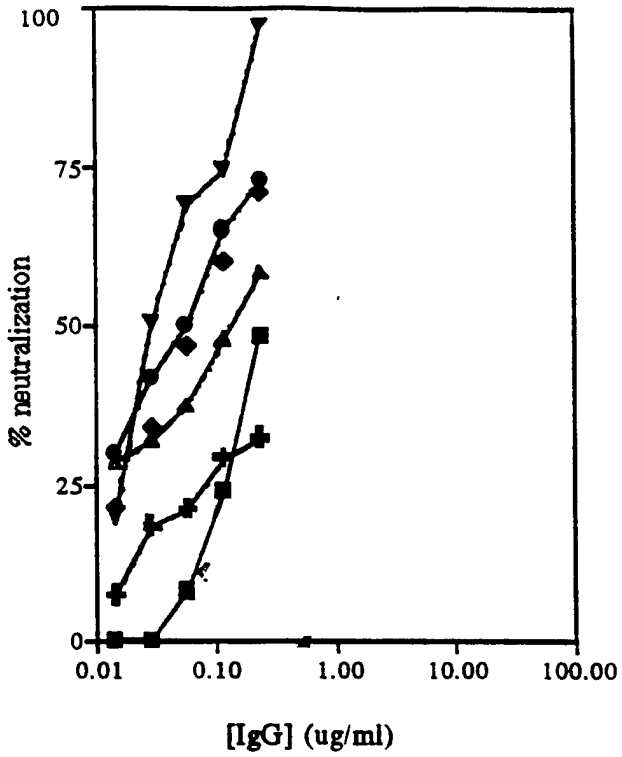

Affinity matrix
gp70 f
V1/V2 peptide
T15K
gp70f -V1/V2
ug IgG recovered
750
500
250
0
757
112
11
300
200
100
0
312
32
nd
150
100
50
0
142
34
6
pH 3
pH 1
GuHCl
Eluate

FIGURE 3

A


100
75
50
25
0
% neutralization
0.01
0.10
1.00
10.00
100.00
[IgG] (ug/ml)

B

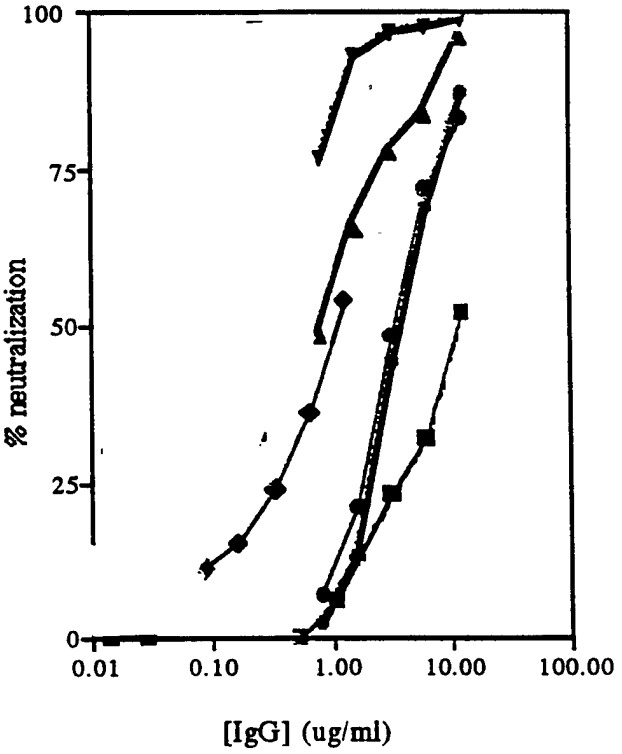
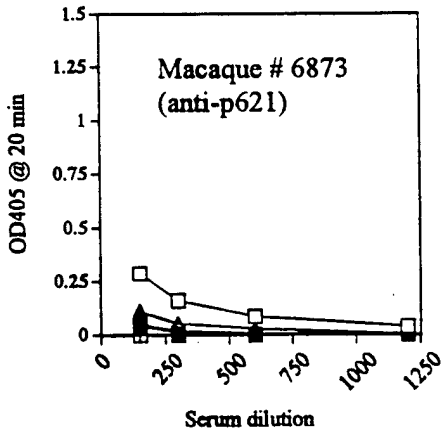

100
75
50
25
0
% neutralization
0.01
0.10
1.00
10.00
100.00
[IgG] (ug/ml)

FIGURE 4

A



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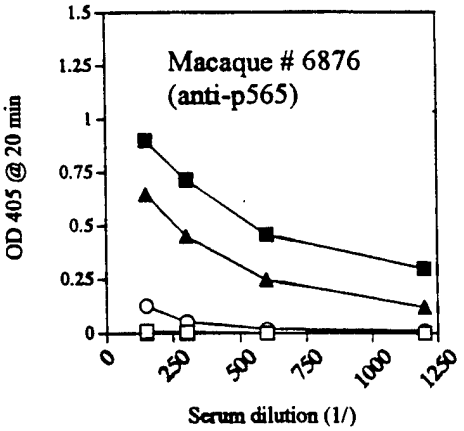

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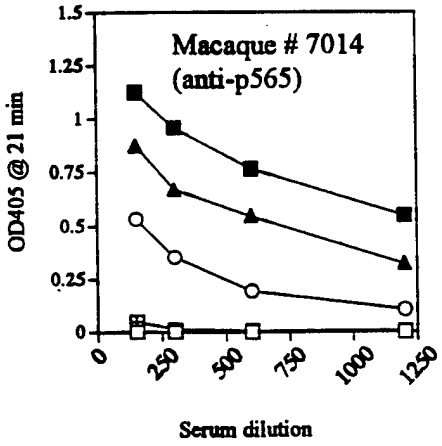

D

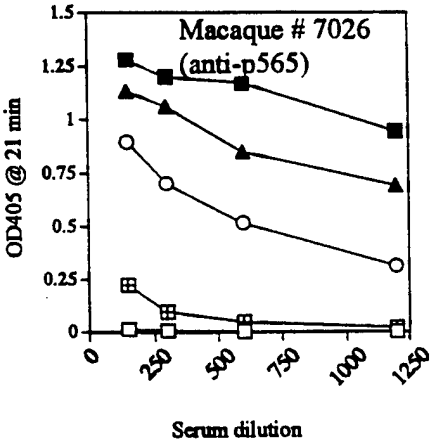

FIGURE 5